USING CHAIN ELONGATION OF CARBOXYLIC ACIDS TO INACTIVATE *ASCARIS* EGGS AND PROVIDE NEW METHODS FOR SANITATION WASTE TREATMENT

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USING CHAIN ELONGATION OF CARBOXYLIC ACIDS TO INACTIVATE *ASCARIS* EGGS AND PROVIDE NEW METHODS FOR SANITATION WASTE TREATMENT

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Improving global coverage of sustainable and safely managed sanitation requires innovative solutions for safely treating waste while saving and recovering resources, and pathogen inactivation is an important component of these solutions. In this dissertation, I present four studies that demonstrated the feasibility of biologically producing carboxylic acids within human fecal material (HFM) as a means of inactivating pathogens. In all studies I use eggs from the *Ascaris suum* roundworm as indicators of pathogen inactivation because *Ascaris* eggs are widely believed to be the most resistant pathogens to sanitation waste treatment processes. I found that HFM is a suitable substrate for carboxylic acid production and chain elongation, and I demonstrated that the microbial community naturally residing in HFM is capable of performing this fermentation. I also found that carbohydrate-rich food waste can be co-fermented with HFM to further increase carboxylic acid concentrations, reduce pH, and promote faster inactivation of *Ascaris* eggs. In addition, I conducted several studies to better understand the parameters that control *Ascaris* inactivation in this system. I demonstrated that only the undissociated form of carboxylic acids is effective for inactivating *Ascaris* eggs, and pH is critical for controlling the fraction of carboxylic acids that are in the undissociated form. However, pH does not directly affect *Ascaris* inactivation on its own. Small changes in mesophilic temperatures and the presence of oxygen also have a strong impact on inactivation rates. In a study testing the effect of temperature without the presence of carboxylic acids, I showed that current guidelines for thermal inactivation of *Ascaris* eggs are overly

conservative, particularly under aerobic conditions, and I developed a new time-temperature relationship for *Ascaris* inactivation at temperatures between 34°C-45°C. In a separate study, I developed a logistic regression model to predict *Ascaris* inactivation as a function of *n*-butyric acid concentration, *n*-valeric acid concentration, *n*-caproic acid concentration, exposure time, and temperature. This model can be used to provide preliminary predictions for operating conditions required in sanitation waste treatment systems.

BIOGRAPHICAL SKETCH

Lauren Alexandra Harroff grew up in Concord, North Carolina with the loving support of her parents, stepparents, twin sister, and later two younger brothers. She attended Clemson University as a member of the National Scholars Program and the Calhoun Honors College, and she received a Bachelor of Science in Biosystems Engineering in 2012. Her incredible mentors and professors at Clemson University helped Lauren pursue diverse interests and opportunities including researching algae biofuels and studying abroad in England, the Netherlands, and South Africa. While in South Africa, Lauren was encouraged to apply for a Fulbright fellowship, which ultimately set her on her current path.

During her final year at Clemson Lauren applied for a Fulbright research fellowship in Uganda and to PhD programs in Biological and Environmental Engineering. She first met Prof. Lars Angenent during this time, but she decided to delay her PhD studies after receiving the Fulbright fellowship. Lauren moved to Kampala, Uganda in September 2012 and lived there for nine months. She worked with the Centre for Research in Energy and Energy Conservation (CREEC) and Makerere University to study the feasibility of using small-scale anaerobic digesters to produce cooking gas for individual families. She worked on many aspects of the project including the impact of digester use on presence of manure pathogens, monitoring indoor air quality in kitchens, and interviewing households about challenges they faced in maintaining digesters. She is forever grateful for her time in Uganda and for the profound impact it has had on her life. She met several lifelong friends; learned how to play ultimate frisbee (which later led her

to her fiancé Jeff); found new independence and confidence in herself; and discovered how to blend her interests into a meaningful career.

Lauren contacted Lars again as her time in Uganda was wrapping up, and she joined him in Ithaca in the fall of 2013 for a six-month position as a research technician to work on the project that would eventually evolve into her PhD dissertation. In 2014 Lauren received a National Science Foundation (NSF) Graduate Research Fellowship and an NSF Integrative Graduate Education and Research Traineeship (IGERT) in Cross-Scale Biogeochemistry and Climate, and she officially decided to stay with Lars at Cornell University to complete her PhD. During her time at Cornell, Lauren has immersed herself in the field of global water, sanitation, and hygiene (WaSH). It was particularly important to her to collaborate directly with practitioners in the field, and she developed a strong relationship with Sanergy, a sanitation enterprise in Nairobi, Kenya. She completed field trials for her dissertation with them and worked on additional projects with their research and development and engineering teams that gave her valuable practical experience in the field. After completing her PhD, Lauren plans to continue working on research and development for water and sanitation projects while working with a government agency, non-profit organization, or social enterprise.

This dissertation is dedicated to the pursuit of a more equitable and sustainable world, which can only be accomplished through the sharing of knowledge, ideas, and values among our global community.

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Second, I would like to express sincere gratitude for all members of the Angenent lab, past and present. The Angenent lab has provided a community of helpful, inspiring, and brilliant friends and colleagues that I have leaned on throughout my PhD. While I appreciate everyone in the Angenent lab, Catherine Spirito, Leo Kucek, and Jiajie Xu deserve special acknowledgement. They made the Morrison lab a warm and happy place that I truly enjoyed going to every day. I also thank Catherine and Leo for the advice, proofreading, bouncing of ideas, and R help that they have continued to offer long after

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In addition, there are many people who have contributed directly to the work in this dissertation and deserve acknowledgement. From Sanergy, I thank Tim Egan, Emily Wangolo, Ani Vallabhanenei, and David Auerbach for allowing me to work with Sanergy and for broadening my experience and perspective on developing meaningful sanitation solutions; Davis Ireri, Jimmy Kirui, Daniel Kyalo, Eunice Muthui, Clifford Odhiambo, and Ricky Ojwang for assistance in planning, set-up, sampling, and lab analysis for the field-scale trial; and the entire NTCO team for the community they provided during my time in Nairobi. From Cornell, I thank Lucinda Li for assistance with *Ascaris* experiments and counting thousands of *Ascaris* eggs while I was in Nairobi; Lynn Johnson from the Cornell Statistical Consulting Unit, without whom Chapter 5 would not exist; and Jeff Carmichael, Theresa Lagasse, Brenda Marchewka, and Peggy Stevens for their kind assistance to me and all of the graduate students in Riley Robb.

I also acknowledge funding from an Academic Venture Fund grant from the Atkinson Center for a Sustainable Future; a National Science Foundation (NSF) Integrative Graduate Education and Research Traineeship (IGERT) in Cross-Scale Biogeochemistry and Climate; a travel grant from the same NSF IGERT program that allowed me to travel to Kenya and establish a collaboration with Sanergy; a fellowship from the NSF Graduate Research Fellowship Program (GRFP); a travel grant from the

Mario Einaudi Center for International Studies; and several conference grants from the Cornell Graduate School.

I also owe tremendous thanks to my family who guided me to this point and supported me throughout this process—to my dad John Harroff, who assumed I would get a PhD long before I did; to my mom Lori Walker, who reminded me to take breaks and have fun; to my sister Lindsay Harroff, who has helped me through every major decision and moment in my life; to Margaret Harroff and Jeff Walker for their love and support; and to my brothers Braiden and Carson Walker who remind me that it's cool to study poop. Finally, my deepest love and gratitude to my fiancé and life partner Jeff Trondsen for gamely learning more about parasites and toilets than any person should know; keeping me sane and happy through stressful moments; knowing exactly when to encourage me to keep going and when to go for a hike together instead; bringing lab equipment to Kenya and helping me fix an autosampler; and for his love and kindness every day.

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CHAPTER 1

INTRODUCTION

1.1 Motivation and objectives

The Sustainable Development Goals aim to achieve universal safely managed sanitation by 2030. Achieving this goal requires 4.5 billion people to gain access to hygienic sanitation facilities and a means of safely managing excreta through containment or treatment (WHO and UNICEF, 2017). Although sewers connected to wastewater treatment plants are the default mechanisms for transport and treatment of human excreta in many parts of the world, these systems are impractical due to high costs, environmental factors, population density, land rights, and many other challenges. Alternatively, waste treatment systems that incorporate resource recovery from human excreta provide a means for reducing both treatment costs and human impact on the environment. Examples of resource recovery processes include anaerobic digestion to produce energy from methane; composting to provide soil amendments and nutrients; and production of black soldier fly larvae (*Hermetia illucens*) as animal feed. Pathogen inactivation is a critical component of these systems to protect workers who handle excreta during resource recovery processes and to protect the end users of the products. However pathogen inactivation in these processes requires either long treatment times (as in the case of composting) or additional treatment steps that require expensive inputs such as commercial chemicals or heat to produce high temperatures. The development of new sanitation technologies that effectively remove pathogens from human excreta are therefore needed to address the global sanitation challenge and to transform conventional resource-intensive waste treatment processes.

In this dissertation I discuss the prospect of using *in-situ* production of carboxylic acids to inactivate pathogens in human fecal material (HFM) and to create safe re-use products. The process utilizes open cultures of bacteria (microbiomes) found naturally in HFM to convert the complex organic molecules of HFM to short- and medium-chain carboxylic acids that are toxic to pathogens. I focused strictly on the solid waste fraction (HFM) because the majority of pathogens found in human excreta are in the feces (Feachem et al., 1983), and urine-diverting slabs can be implemented relatively easily in both dry and flush toilets (Tilley et al., 2014). For pathogen inactivation, I used eggs from the *Ascaris* roundworm as indicators because they are among the most resistant pathogens to disinfection processes found in human excreta (Arfaa, 1978; Bowman et al., 2000; Feachem et al., 1983; U.S. EPA, 2003). The objectives of this work were to: 1) demonstrate that HFM could be used as substrate for production and chain elongation of carboxylic acids; 2) determine carboxylic acid concentrations and environmental conditions that are required for *Ascaris* inactivation; and 3) demonstrate the feasibility of this system in a field-scale trial.

1.2 Organization and summary of chapters

Chapter 2 provides a literature review with primary emphasis on pathogen inactivation by carboxylic acids. Specifically, attention is given to: 1) the structure of the *Ascaris* egg and its contribution to their resistance to inactivation; 2) known inactivation mechanisms for *Ascaris* that are relevant to understanding carboxylic acid inactivation; and 3) inactivation of other microorganisms and pathogens by carboxylic acids. Previous work on production and chain elongation of carboxylic acids is also briefly summarized with

particular emphasis on waste streams that have previously been used as substrates and the concern of product inhibition.

Chapter 3 details initial laboratory work that established the feasibility of the central goal of this dissertation: to inactivate pathogens in HFM through production and chain elongation of carboxylic acids. Through batch 1-L fermentation experiments, I demonstrated that HFM is a suitable substrate for production and accumulation of carboxylic acids, including *n*-butyric acid (C4) and *n*-caproic acid (C6). I also showed that *n*-butyric acid and *n*-caproic acid can inactivate *Ascaris* eggs at the concentrations that were found in the fermentation experiments, and I developed relationships for exposure times and concentrations of each acid required to achieve > 99.9% inactivation at 30°C. In Chapter 3 I also concluded that carboxylic acids with longer hydrocarbon chains are more effective inactivating agents than those with shorter hydrocarbon chains and that all carboxylic acids must be in the undissociated form to cause inactivation. The fraction of total acid concentration that is in the undissociated form is controlled by pH, where a pH less than the pKa of the acid (~4.8) is desired. Finally, I found that pH has no impact on *Ascaris* viability beyond controlling the fraction of the undissociated form.

Chapters 4 and 5 focus strictly on inactivation of *Ascaris* eggs. In Chapter 4, the effect of exposure to mesophilic temperatures (≤ 45°C) is explored without the presence of other inactivating compounds. I combined experimental data with evidence from literature to prove that current guidelines for thermal inactivation of *Ascaris* are unrealistically conservative, and I developed a new relationship for time and temperature combinations that cause > 99.9% inactivation of *Ascaris* eggs. I also showed that the presence of oxygen can greatly decrease required exposure times for thermal inactivation

of *Ascaris* eggs and may be relevant to other inactivation mechanisms as well. In Chapter 5, I developed a predictive model to estimate expected *Ascaris* viability as a function of concentrations of three carboxylic acids (*n*-butyric acid, *n*-valeric acid, and *n*-caproic acid), exposure time, and temperature. The model likely overpredicts inactivation rates in some cases, but it can be used to give initial estimates of conditions that will be required in sanitation systems to achieve pathogen inactivation.

Chapter 6 presents work that I performed in collaboration with Sanergy, which is a sanitation enterprise based in Nairobi, Kenya. My goal for this work was to demonstrate that the proposed sanitation treatment system is feasible at a larger scale outside of the lab and to understand some of the operating conditions that would be required if it were implemented at scale. Through laboratory experiments, I demonstrated that the natural community of bacteria found in HFM performed as well or better than alternative inocula for producing carboxylic acids. This important finding eliminates one potential logistical and economic challenge for implementation at scale. I also demonstrated that carbohydrate-rich food waste (specifically banana waste) could be co-fermented with HFM to reduce pH, increase the concentration of undissociated carboxylic acids, and cause > 99% *Ascaris* inactivation within 15 days. When scaling up to 45-L batch field trials, I found that warm temperatures (~27°C or more) were required to achieve adequate fermentation rates, and I discovered that food waste is rapidly degraded to reduce pH while also providing additional substrate for carboxylic acid production. The addition of food waste during the field trial resulted in higher concentrations of *n*-caproic acid (maximum of 43 mM) than we observed in any previous trials, which is extremely promising for further development of this work.

Finally, Chapter 7 provides conclusions, recommendation for future work, and an overview of the outlook for carboxylic acid fermentation to be utilized in sanitation processes.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Providing global safe and equitable sanitation poses both a challenge and an opportunity. From the public health perspective, human fecal material (HFM) must be treated to prevent pathogens from contaminating food and waterways. With a lack of traditional wastewater infrastructure in many developing countries, this task can be challenging but also provides an opportunity to develop new waste treatment systems that are superior to conventional sewage and wastewater treatment. Specifically, to promote sustainable growth in emerging economies, bold new proposals are needed for HFM treatment systems which: 1) effectively remove pollutants and pathogens from HFM; 2) are economically sustainable; and 3) recycle valuable nutrients as safe and affordable fertilizers.

Lack of adequate sanitation facilities leads to many health concerns, particularly in young children who are more susceptible to fecal-oral transmission of disease. Diarrhea, which is often a result of exposure to pathogens in HFM, is estimated to account for 21% of all deaths in children under age five in developing countries (Kosek et al., 2003). One pathogen of particular interest is *Ascaris lumbricoides,* an intestinal helminth that infects approximately 1.3 billion people around the world (De Silva et al., 1997). Due to its unique life cycle and egg structure, *A. lumbricoides* can remain dormant in the soil for several years and remain infective once ingested by a person (Brudastov et al., 1970). The chemical composition of the eggshell protects the enclosed larva from most chemical disinfectants and makes *A. lumbricoides* a useful indicator

organism for testing treatment methods for HFM (Fairbairn and Passey, 1955; U.S. EPA, 2003).

Carboxylic acids have previously shown the ability to permeate *Ascaris* eggs and inhibit development of the larvae (Barrios et al., 2007; Butkus et al., 2011; Rojas-Oropeza et al., 2016; Takeyama, 1951). Furthermore, carboxylic acids with longer carbon chains are known to be more toxic to *Ascaris* eggs and other pathogens than carboxylic acids with shorter carbon chains (Abdul and Lloyd, 1985; Butkus et al., 2011; Liu et al., 2013; Royce et al., 2013; Takeyama, 1951). In recent years, the use of mixed cultures of microbial communities (microbiomes) to produce and chain elongate carboxylic acids from organic waste streams has been investigated as part of the carboxylate platform (Agler et al., 2011).

In this dissertation, a novel sanitation system is proposed. HFM can be fermented anaerobically to produce and chain elongate carboxylic acids through the carboxylate platform. The accumulated carboxylic acids in the HFM will then inactivate pathogens, rendering the material safe to handle and apply to land or to feed into a second processing step to produce other value-added products, such as an anaerobic digester to produce biogas for energy. The literature review presented here provides relevant background information including: 1) the structure and inactivation mechanisms of *Ascaris* eggs; 2) the toxicity of carboxylic acids to *Ascaris* eggs and other pathogens; and 3) the production and chain elongation of carboxylic acids through the carboxylate platform.

2.2 Ascaris

Ascaris lumbricoides is an intestinal helminth (parasitic worm) that is spread by fecal-oral transmission. While most infections are asymptomatic, heavier worm burdens can cause symptoms that range from moderate discomfort to death. The most common mild symptoms are growth deficiency, decline in physical fitness, and reduction of cognitive ability (De Silva et al., 1997; O'lorcain and Holland, 2000). More acute symptoms and complications include intestinal obstruction, appendicitis, liver damage, and perforation of the intestine, all of which can ultimately cause death. Symptoms are generally more serious in children than in adults (De Silva et al., 1997; Feachem et al., 1983).

Adult *A. lumbricoides* worms reside in the small intestine, where a female may shed up to 200,000 eggs per day (Feachem et al., 1983). These eggs are passed through feces to the environment. In preferred conditions (22°C-33°C, moist, shaded, and aerobic), fertilized eggs will develop into viable larvae in 10-15 days. Under non-ideal growth conditions, the eggs may remain dormant in the soil for up to ten years, after which they can still commence growth and infectivity when exposed to more preferred conditions (Brudastov et al., 1970; Feachem et al., 1983). The larvae will hatch and develop into adult worms only after the eggs are ingested by a human. The ability to survive for years outside of a host and then recommence growth combined with the impermeability of the egg make *A. lumbricoides* one of the most resistant pathogens to consider with sanitation processes (Feachem et al., 1983).

Both *Ascaris lumbricoides* and *Ascaris suum* will be referenced in this text. *A. suum* is a helminth infective to pigs with a similar egg structure to *A. lumbricoides.* Due to its low risk of infectivity to humans and greater abundance in the environment, *A.*

suum is often used as a reliable surrogate for *A. lumbricoides* when testing egg viability and inactivation (Feachem et al., 1983). Throughout this text, the specific species *A. lumbricoides* and *A. suum* will be designated when relevant. Otherwise, the genus name *Ascaris* will be used for statements believed to apply to both species (e.g., when experiments have been performed on *A. suum* but are intended to apply to *A. lumbricoides* or when discussing egg structure that applies to both species).

2.2.1 Ascaris *egg structure*

Ascaris eggs are oval-shaped, measure approximately 50 μm by 75 μm, and contain four distinct layers (**Figure 2.1**). From outermost to innermost, these layers are: 1) a mammilated albuminous outer layer secreted by the uterine cells of the female worm; 2) a lipoprotein vitelline layer; 3) a thick chitinous layer that provides structural support for the egg shell; and 4) a lipid layer, sometimes called the ascaroside layer (Bowman and Liotta, 2012; Wharton, 1980).

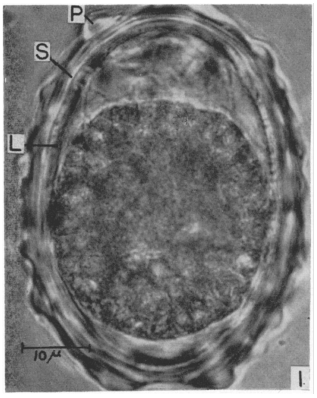

Figure 2.1 Layers of an *Ascaris* egg. Microscopy image of an *A. suum* egg taken from a 1956 study by Rogers (Rogers, 1956). The egg layers are labeled as: the mammilated protein outer layer (P); chitinous shell (S); and lipid layer (L). Rogers argues that the lipoprotein vitelline layer is actually part of the protein outer layer, giving the egg only three layers instead of four (Rogers, 1956).

The high resistance of *Ascaris* eggs to many chemical disinfectants is primarily attributed to the innermost lipid layer (Fairbairn and Passey, 1955; Khanna and Yadav, 2004; Wharton, 1980). The composition of the layer is 75% lipid and 25% protein, with the lipid portion controlling the unique permeability characteristics of the eggs (Fairbairn, 1957; Wharton, 1980). The exact composition of the lipid fraction has been studied and discussed since the early 1900s. In 1913, Fauré-Frémiet (1913) first suggested the major component of the membrane to be "ascaryl alcohol", which had previously been isolated from reproductive tissues of adult *Ascaris* worms. Fairbairn and Passey (1955) later confirmed the presence of ascaryl alcohol through the first chemical analysis of the lipid membrane. For this study, the three outer layers of *A. lumbricoides* eggs were removed, and it was noted that the eggs remained resistant to many chemicals including 2 N hydrochloric acid, 2 N nitric acid, 2 N sodium hydroxide, 0.5 N ammonium hydroxide, 3.3 N formaldehyde, and 4 N sodium chloride. This portion of the study showed that the inner membrane provides the greatest resistance to inorganic chemical disinfectants. The authors then collected the inner membrane and confirmed ascaryl alcohol, an unsaponifiable lipid, to be its primary component (Fairbairn and Passey, 1955). Fouquey et al. (1957) later determined the structure of ascaryl alcohol to be a series of closely related esterified glycosides, which were named ascarosides. Ascarosides consist of a glycine, 3,6-ideoxy-L-arabinohexose (sometimes called ascarylose), and a secondary alcohol chain containing 21 to 33 carbons (Wharton, 1980). The lipid solubility traits of ascarosides make the inner

membrane permeable to organic disinfectants, such as carboxylic acids, while remaining resistant to many inorganic chemicals (Khanna and Yadav, 2004).

The nomenclature of the innermost egg layer and the chemical classification of ascarosides have been inconsistent in older literature. Fairbairn and Passey (1955) referred to the innermost membrane as the vitelline membrane. However, subsequent researchers have agreed that it is more correct to name the third layer directly outside the chitin layer the vitelline membrane and to name the innermost layer the lipid membrane or lipid layer (Bird, 1971; Foor, 1967; Wharton, 1980). Some researchers have also suggested that ascarosides possess similar solubility properties as lipids, but that ascarosides are not true lipids themselves (Barrett, 1976; Fouquey et al., 1957; Khanna and Yadav, 2004). The preferred classification is that ascarsosides are a type of lipid and the primary component of the lipid portion of the lipid membrane (Jezyk and Fairbairn, 1967; Tarr and Schnoes, 1973; Wharton, 1980).

Increased temperature is one variable that has been shown to increase the permeability of *Ascaris* eggs. A study by Wharton (1979) tested water loss during desiccation of *A. lumbricoides* eggs at constant relative humidity and varying temperature. The study found that the rate of water loss increased exponentially with increasing temperature over the range of 16.5°C–45°C. While some increased loss could be explained by the increase of water vapor pressure with increased temperature, the rate of increase in water loss was too great to be explained only by this factor. Wharton also found that after exposure to temperatures of 63°C–65°C, the eggshell became completely permeable to water (Wharton, 1979). This temperature corresponds

to the previously reported melting point of 70°C for the inner lipid membrane of the egg (Fairbairn and Passey, 1955).

2.2.2 Ascaris guidelines

Due to the impermeability of the lipid membrane and effect of *A. lumbricoides* infections on public health, *Ascaris* eggs are an important consideration in the treatment of HFM for land application. The U.S. Environmental Protection Agency (EPA) and World Health Organization (WHO) both provide recommendations and guidelines for the application of human waste biosolids to land (U.S. EPA, 2003; WHO, 2006). For biosolids application in the United States, the EPA 40 CFR Part 503 regulations must be met (U.S. EPA, 2003). Five classes of pathogens are considered for the acceptable treatment of biosolids: bacteria, enteric viruses, enteroviruses, protozoa, and helminths. Of these, helminths are considered the most resistant to both chemical disinfectants and high temperatures. *Ascaris* spp. are known to be the hardiest of the helminths and are therefore used as indicator organisms for the inactivation of all other helminths (U.S. EPA, 2003).

The EPA characterizes two classes of biosolids: Class A and Class B. Class B biosolids are not considered to be completely free of pathogens and are therefore restricted to application on only certain types of land. To meet Class B standards, no reduction in viable helminth ova is required. Class A standards require pathogen loads to be reduced below detectable limits, defined as: 1) less than 3 most probable number (MPN) per 4 g total solids (TS) for *Salmonella* spp.; 2) less than 1 plaque forming unit (PFU) per 4 g TS for enteric viruses; and 3) less than 1 viable helminth ova per 4 g TS.

Class A standards can be met by adhering to one of two accepted treatment methods (high temperature and time requirements or alkaline treatment at high temperature) or by continuous monitoring of pathogen loads and pathogen inactivation. The monitoring processes for helminths and enteric viruses are particularly difficult and time consuming, making it arduous to meet Class A biosolids standards with alternative treatment methods (U.S. EPA, 2003).

The WHO also outlines guidelines for the treatment of biosolids or sewage sludge that will be applied to land. Unlike the EPA Part 503 guidelines, the WHO recommendations account for both the pathogen load and public health risks posed by those pathogens. In other words, instead of requiring pathogen counts to be reduced below detection, recommended pathogen loads are based on a combination of factors including: 1) survival rates in the outside environment; 2) modes of transmission; 3) the specific health risk posed by each pathogen; and 4) the background burden of disease within a specific population. As stated in the guidelines:

> *Overly strict standards may not be sustainable and, paradoxically, may lead to reduced health protection, because they may be viewed as unachievable under local circumstances and, thus, ignored. The Guidelines therefore strive to maximize overall public health benefits and the beneficial use of scarce resources.* (WHO, 2006)

Microbial reduction targets for excreta applied to land include less than 10^3 *E. coli* per g TS and less than 1 viable helminth ova per g TS, compared to less than 0.25 viable helminth ova per g TS in the EPA Part 503 guidelines (U.S. EPA, 2003; WHO, 2006). A suggested treatment to remove pathogens is thermophilic digestion at 50°C with a retention time of 14 days followed by aerated composting for one month at 55-60°C. This long period of treatment time is suggested to provide a factor of safety, allowing for cool zones below expected treatment temperatures within the material (WHO,

2006). The WHO guidelines also state that if *Ascaris* ova are absent from excreta naturally, there is no need to maintain treatment conditions necessary to inactivate them, which is not the case for the EPA guidelines (U.S. EPA, 2003; WHO, 2006).

2.2.3 Inactivation by temperature

Temperature has frequently been evaluated as a possible treatment method for *Ascaris* eggs, with mixed conclusions. The EPA Part 503 regulations provide four time-temperature relationships that can be used to meet Class A biosolids standards, to be chosen based on the total solids concentration and matrix of biosolids to be treated. For most anaerobic digester conditions and for undiluted HFM, Regime A (for sludges greater than 7% total solids) of the regulations should be used (**Equation 2.1**), where T is temperature in °C and t is required exposure time in days.

$$t_{EPA} = (1.317\times10^{8})\times10^{-0.14T} \qquad \textbf{(Equation 2.1)}$$

Based on these guidelines, sludge heated to 50°C must be maintained at that temperature for at least 13.2 days (**Figure 2.2**). In addition, the relationship is considered uncertain for any temperature below 50°C so treatment below this threshold is not permitted for Class A biosolids. These conservative requirements are based partly on the FDA requirements for treatment of eggnog, a food that is noted to have similar flow characteristics to sewage sludge (U.S. EPA, 2003).

Previously, Feachem et al. (1983) also proposed a relationship between temperature and required treatment time. Based on a compilation of pre-1983 literature, the authors created a plot of temperature versus time and plotted points as either "100% destruction of *Ascaris* eggs" or "less than 100% destruction of *Ascaris* eggs". They then

drew a line so that all points with less than 100% destruction would fall below it. Any time and temperature combination above the line was considered to be in the "zone of safety" where 100% destruction of *Ascaris* eggs could be reasonably assumed. An equation was not originally given to calculate the zone of safety, but it was later derived by Vinnerås et al (2003) (**Equation 2.2**), where T is temperature in °C and t is required exposure time in hours.

$$t_{Feachem} = 177 \times 10^{-0.1944(T-45)} \qquad \textbf{(Equation 2.2)}$$

Based on this equation, a temperature of 50°C requires an exposure time of 19 hours, which is much lower than the 13.2 days required by the EPA (**Figure 2.2**) (Feachem et al., 1983; U.S. EPA, 2003; Vinnerås et al., 2003).

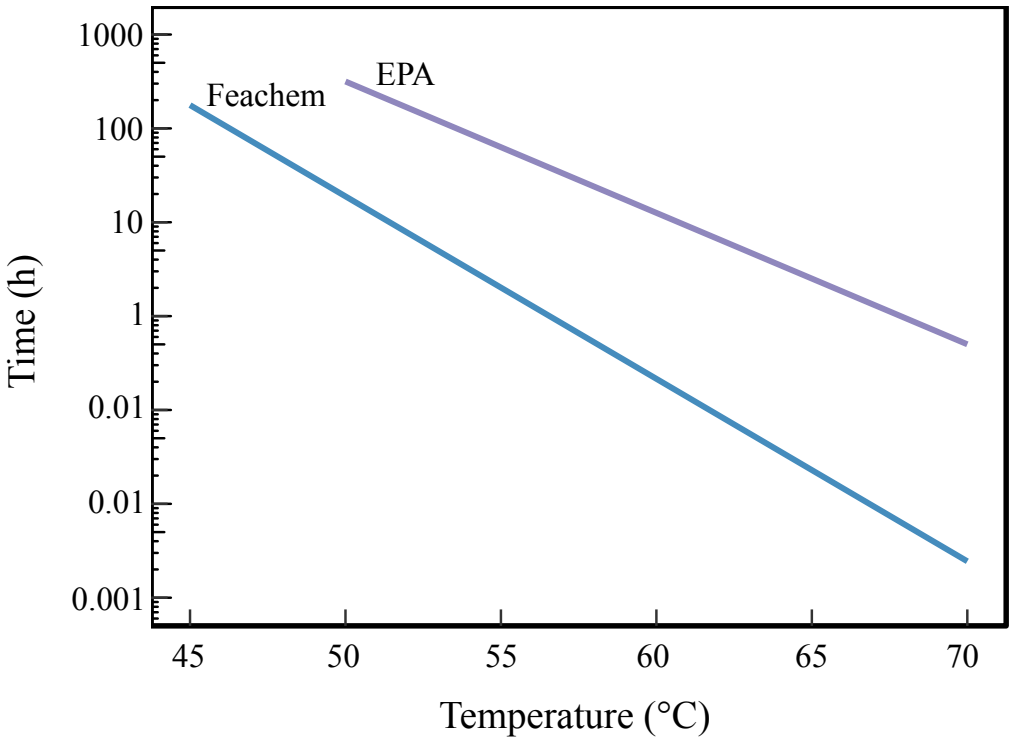

Figure 2.2 Relationships for time and temperature expected to provide adequate *Ascaris* inactivation (Feachem et al., 1983; U.S. EPA, 2003).

More recent literature has continued to demonstrate that the EPA requirements for *Ascaris* inactivation may be overly conservative. Aitken et al. (2005) measured inactivation of *A. suum* eggs in biosolids from a lab-scale continuous anaerobic digester at controlled temperatures ranging between 49°C and 55°C. The study found the relationship between the log concentration of viable *Ascaris* eggs and time to be generally first-order, and it predicted that eggs would be inactivated below detection after two hours at 51°C, compared to 9.5 days required by the EPA regulations or 12 hours expected from the Feachem relationship (Aitken et al., 2005; Feachem et al., 1983; U.S. EPA, 2003; Vinnerås et al., 2003). The decimal reduction time (time required to achieve 90% inactivation) at 49°C was calculated to be 110 minutes, indicating a 3-log reduction could be expected after 5.5 hours. The decimal reduction time at 51°C was 32 minutes, corresponding to a 3-log reduction after 1.6 hours (Aitken et al., 2005). Popat et al. (2010) also investigated inactivation of *Ascaris* eggs in anaerobic digester sludge at temperatures between 51°C and 55°C. The inactivation rates seen here were slower than those observed by Aitken et al. (2005) but still significantly faster than the rates from the EPA and Feachem time-temperature relationships (Popat et al., 2010). Differences between the Popat et al. (2010) study and the Aitken et al. (2005) study were suggested to be due to differences in the composition of the sludge matrix with more protective compounds possibly present in the sludge from Popat et al. (2010) and/or more inhibitory compounds present in the sludge from Aitken et al. (2005).

Mesophilic temperature treatment has generally been cited as insufficient for *Ascaris* inactivation (Feachem et al., 1983; U.S. EPA, 2003), but evidence from

literature and experimental observations suggest that mesophilic temperatures can, in fact, inactivate *Ascaris* eggs. This topic is discussed in depth in Chapter 4 of this dissertation.

2.2.4 Ammonia inactivation

Several studies have investigated ammonia as an inactivating agent for *Ascaris* eggs. These studies provide useful insight for studying inactivation by carboxylic acids because the mechanism for inactivation is believed to be similar (Butkus et al., 2011; Pecson and Nelson, 2005). At high pH (greater than the pKa of 9.3), ammonia exists primarily in the undissociated NH_3 form as opposed to the charged NH_4^+ form. The undissociated form is lipophilic and readily crosses biological membranes, such as the lipid layer of *Ascaris* eggs, where it disrupts the internal pH of the organism or cell. The charged form does not easily cross membranes (Pecson and Nelson, 2005; Warren, 1962). The effects of ammonia concentration, pH, and temperature on *Ascaris* inactivation have been tested. Pecson and Nelson (2005) showed that high pH only affects egg inactivation indirectly by controlling the fraction of ammonia that is in the undissociated NH_3 form. In other words, when the concentration of undissociated NH_3 was held constant at varying pH values, the viability of eggs was not affected. Chapter 3 in this dissertation shows that the same result is true for pH and carboxylic acid inactivation of *Ascaris* eggs.

In contrast to pH, temperature has a significant direct effect on egg inactivation (Ghiglietti et al., 1995; Pecson et al., 2007; Pecson and Nelson, 2005). At temperatures between 40°C and 48°C, inactivation increased significantly with increased temperature

when NH_4Cl concentration and pH were held constant (Pecson and Nelson, 2005). In a similar study with temperatures ranging between 20°C and 50°C, every 10°C increase in temperature was found to significantly decrease the time required to achieve 99% inactivation (t_{99}) (Pecson et al., 2007). Another study of *A. suum* eggs, this time spiked into human feces containing ammonia, quantified a five-fold decrease in inactivation time for every increase of 10°C (Nordin et al., 2009). Fidjeland et al. (2015) combined data from several studies to develop a model for *Ascaris* egg inactivation by ammonia and temperature. They found that the inactivation rate increased with NH_3 activity to the power of 0.7 and that the exposure time needed to achieve a given inactivation rate decreased ten-fold with each 16°C increase in temperature, up to 42°C. Above 42°C, the temperature response increases (Fidjeland et al., 2015). Increased temperature is believed to increase the permeability of the lipid layer in *Ascaris* eggs, which allows undissociated NH_3 to pass through the membrane more easily (Nordin et al., 2009; Wharton, 1979). The same mechanism would likely apply to the transfer of carboxylic acids across the lipid membrane of the egg; therefore, we would expect to see a similar temperature response in relation to carboxylic acid inactivation. The effect of temperature on *Ascaris* inactivation by carboxylic acids is discussed further in Chapter 5 of this dissertation.

The inhibitory concentrations of NH_3 varied widely based on exposure time and incubation temperature and are therefore difficult to summarize briefly; however, some concentrations seemed particularly relevant to this study. First, approximately 340 mg/L (20 mM) undissociated NH_3 was suggested as a threshold concentration for *Ascaris* egg inactivation (Nordin et al., 2009). Below this concentration, no inactivation was

observed irrespective of temperature or incubation time. This number is important when considering inactivation experiments that have been performed on eggs in buffered solution versus those performed directly in sludge or feces. If the ambient concentration of NH_3 in the incubation matrix is above 340 mg/L, ammonia inhibition may contribute to any inactivation observed. Also of particular interest to developing sanitation technologies, Nordin et al. (2009) found that a three-log reduction of *Ascaris* viability could be achieved after six months of storage at 24°C in urine with an NH_3 concentration of approximately 1124 mg/L or 66 mM (a 1:1 dilution of the urine). Similarly, 1.5 months of storage at 34°C in urine diluted no more than 1:3 (NH_3 equal to 680 mg/L or 40 mM) also yielded a three-log reduction in viability (Nordin et al., 2009).

2.3 Toxicity of carboxylic acids

Although relatively little research exists on the toxicity of carboxylic acids to *Ascaris* eggs, significant work has focused on the toxicity to other bacteria and pathogens. Carboxylic acids have been shown to inhibit growth in *Escherichia coli* (Abdul and Lloyd, 1985; Royce et al., 2013; Skřivanová et al., 2006), *Salmonella* spp. (Kunte et al., 2004; Salsali et al., 2006; Skřivanová et al., 2006), *Saccharomyces cerevisiae* (Liu et al., 2013), *Shigella dysenteriae* (Kunte et al., 2004), *Vibrio chloerae* (Kunte et al., 2004), and *Clostridium perfringens* (Skřivanová et al., 2006).

2.3.1 Mechanisms for carboxylic acid inhibition

Because carboxylic acids are weak acids, they exist in equilibrium between the undissociated acid and the negatively charged conjugate base. The Henderson-Hasselbalch equation (**Equation 2.3**) can be used to calculate the fraction of the total concentration that exists in either form.

$$pH = pK_a + log\frac{[A^-]}{[HA]} \qquad \textbf{(Equation 2.3)}$$

When the pH is less than the pK_a of the carboxylic acid, the undissociated acid form (HA) will dominate, while the charged conjugate base (A^-) will dominate when the pH is above the pKa (Clugston and Flemming, 2000). Consistently across the literature, carboxylic acids were found to be more inhibitory at lower pH values, when a greater fraction is in the undissociated acid form (Fukushi et al., 2003; Henry et al., 1983; Kunte et al., 2000; Liu et al., 2013; Royce et al., 2013). The undissociated acid can freely cross the plasma membranes of cells, while the conjugate base cannot. Once in the more neutral environment of the cytoplasm, protons dissociate from the acids to establish a new equilibrium, causing a toxic accumulation of both protons and anions inside the cell (Brul and Coote, 1999).

Carboxylic acid toxicity has been attributed to several mechanisms. Most commonly, the release of free protons by the carboxylic acid inside the cell dissipates the transmembrane proton gradient between the cell and external environment (Herrero et al., 1985; Nicolaou et al., 2010). In an attempt to maintain the proton gradient, the cell uses the energy from the breakdown of ATP to pump protons out of the cell via ATPase, thereby limiting the ATP energy available for cell growth (Herrero et al., 1985; Russell, 1992). When the concentration of free protons becomes too high to remove

from the cell, the pH reduction of the cytoplasm also causes a breakdown of basic cell metabolism and inhibits necessary enzymes (Booth, 1985; Russell, 1992).

Another inhibition mechanism has been proposed through intracellular accumulation of the anionic conjugate base (Carpenter and Broadbent, 2009; Russell, 1991, 1992). Russell (1991, 1992) argued that carboxylic acids are poor candidates for uncoupling the proton gradient of the cell because the conjugate base cannot cross the cell membrane back to the external environment. More potent uncouplers are lipophilic enough in both the acid and base forms to cross the cell membrane. When the external pH is low, the undissociated form dominates and crosses into the cell. Once in the higher pH environment of the cytoplasm, the proton dissociates. The proton is pumped out of the cell via ATPase, and the anion passes back through the membrane due to equilibrium principles. Once in the low pH environment outside the cell, the anion is re-protonated. In this manner, the same molecule can cycle in and out of the cell, using considerable ATP energy that would otherwise be used for cell growth and metabolism. Carboxylic acids, on the other hand, can contribute a maximum of one proton inside the cell because the anion becomes trapped insid. Therefore, Russell (1991, 1992) hypothesized that anion accumulation contributed more to carboxylic acid toxicity than did dissipation of the proton gradient. Carpenter and Broadbent (2009) later suggested that anion accumulation would increase cell osmolarity and ultimately cause lethal turgor pressure.

Two recent studies have focused on the impact of carboxylic acids on the cell membrane itself (Liu et al., 2013; Royce et al., 2013). The studies examined the effects of *n*-caprylic acid (C8) on the fluidity and integrity of the cell membranes in *E. coli*

(Royce et al., 2013) and the yeast *S. cerevisiae* (Liu et al., 2013). Both studies found that membrane fluidity increased and membrane integrity (as measured by the leakage of intracellular magnesium from the cell) decreased with increased exposure to *n*-caprylic acid. The authors proposed that carboxylic acids are partially soluble in the membrane. Instead of passing through the membrane into the cytoplasm, some acids may integrate into the membrane and increase its fluidity by reducing the hydrophobic interactions of the fatty acid tails of the membrane phospholipids. Membrane damage can cause growth inhibition through the loss of intracellular metabolites and the uptake of harmful chemicals that would normally be excluded by the membrane (Liu et al., 2013; Royce et al., 2013).

2.3.2 Chain length of carboxylic acids and microbial inhibition

Several studies have shown that short-chain carboxylic acids (SCCAs, one to three carbons) and medium-chain carboxylic acids (MCCAs, four to eight carbons) become more toxic as the length of the carbon chain increases (Abdul and Lloyd, 1985; Freese et al., 1973; Liu et al., 2013; Royce et al., 2013; Woolford, 1975). The toxicity of long-chain carboxylic acids (LCCAs, more than eight carbons) is variable depending on the microbe. A study by Woolford (1975) determined minimum inhibitory concentrations (MICs) of carboxylic acids with one to twelve carbons for 34 different microbial cultures including: yeast, spore-forming bacteria, fungi, lactic acid bacteria, and generic gram-negative and gram-positive bacteria. For the gram-positive bacteria, MIC consistently decreased as chain length of the acid increased. For the gram-negative bacteria, MIC followed the same trend for most SCCAs and MCCAs. Then MIC

increased again for carboxylic acids with more than six or eight carbons. Spore-forming bacteria, fungi, and lactic acid bacteria generally followed the same trends as gram-positive bacteria (Woolford, 1975).

In another study, Abdul and Lloyd exposed *E. coli*, a gram-negative bacteria, to varying concentrations of acetic acid (C2), propionic acid (C3), *iso*-butyric acid (C4), *n*-heptanoic acid (C7), *n*-caprylic acid (C8) and *n*-capric acid (C10). For all SCCAs and MCCAs, increasing chain length caused increased inhibition of *E. coli,* but the trend did not hold for *n*-capric acid. At concentrations of 2.5 mM, *n*-heptanoic acid and *n*-caprylic acid completely inhibited *E. coli* growth for two of the three strains tested. No growth inhibition was observed for *n*-capric acid at the same concentration. It should be noted that pH was not strictly controlled or measured in this study. Cultures were grown at pH 8.0, but the pH was seemingly not adjusted after the carboxylic acids were added to the growth medium. Therefore, all results are reported as the total concentration of undissociated acid plus conjugate base, and the exact concentrations of undissociated carboxylic acids are unknown (Abdul and Lloyd, 1985).

Two mechanisms for the resistance of gram-negative bacteria to LCCAs have been proposed: 1) gram-negative bacteria are able to metabolize LCCAs, thereby preventing them from accumulating to toxic concentrations; and 2) the lipopolysaccharide outer leaflet of the outer membrane in gram-negative cells inhibits transport of larger hydrophobic carboxylic acids into the cytoplasm of the cell (Freese et al., 1973; Nikaido, 2003; Overath et al., 1969). However, the resistance of gram-negative bacteria to LCCAs is inconsistent. A more recent study with *E. coli* reported that *n*-capric acid completely inhibited growth at a total concentration of 20 mM, while

40 mM (total concentration) was required for *n*-caprylic acid and *n*-caproic acid. The pH for this study was reported as a range between 6.5 and 7.0, which makes a comparison of undissociated acid concentrations difficult. Using the Henderson-Hasselbalch equation and given pH range, the possible inhibitory undissociated acid concentrations were calculated as: 0.16-0.49 mM for *n*-capric acid; 0.31-0.96 mM for *n*-caprylic acid; and 0.30-0.94 mM for *n*-caproic acid. At an average pH of 6.75, the inhibitory concentrations were 0.3 mM for *n*-capric acid and 0.5 mM for *n*-caprylic and *n*-caproic acids. This result indicates that the ten-carbon *n*-capric acid is more toxic to *E. coli* than *n*-caprylic or *n*-caproic acids, which contradicts the findings of Woolford (1975) and Abdul and Lloyd (1985). However, if the pH was not consistent between treatments, the results may indicate a different trend. The authors also noted that the increased inhibitory effect of *n*-capric acid (compared to *n*-caprylic and *n*-caproic acids) was less severe than observed in a similar study with yeast (Liu et al., 2013).

Salsali et al. (2006) tested inhibitory effects of acetic, propionic, and *n*-butyric acids with *Salmonella* spp., which is also a gram-negative bacteria, and reported that carboxylic acids with shorter carbon chains were more toxic than those with longer chains at equal concentrations (by mass). This trend is the opposite of what we expect for chain lengths of two to four carbons. When concentrations were converted to a molar basis, however, the reported trend did not hold (Salsali et al., 2006). The molar concentration provides a more accurate comparison than mass concentration because each carboxylic acid molecule contributes only one hydrogen ion when it dissociates inside the cell, regardless of the differing mass of the molecules. The *Salmonella* spp. reduction observed was also very small at the tested concentrations and made

comparisons difficult. The same study more reliably demonstrated that inactivation rates increased with increasing temperature when the acid type and concentration were held constant (Salsali et al., 2006). A more recent study by Riungu et al. (2018) also used mass concentration instead of molar concentration and concluded that carboxylic acids with shorter chains were more toxic to *E. coli*.

2.3.3 Carboxylic acids and Ascaris

Although studies exist in the literature that reference carboxylic acid inactivation of *Ascaris* eggs, in-depth investigation of the required conditions and concentrations of acids remain limited. The earliest study to my knowledge already suggested that the ovicidal action of carboxylic acids increases with an increase in the number of carbons, but few details from this work are known due to the abbreviated presentation of results and discussion and the apparent loss of nuances in the translation (Takeyama, 1951). Another study found that acetic acid reduced *Ascaris* viability, but the 30-minute exposure time was too short to achieve more than an 81% inactivation, and no information about temperature was provided (Barrios et al., 2007). In a third study, *Ascaris* eggs were claimed to be inactivated after 56 days of exposure to lactic acid that had been produced from fermented manure; however, the control eggs in this study were also completely inactivated, likely due to the 37°C incubation temperature (Scheinemann et al., 2015). Another study tried to simulate carboxylic acid concentrations that would be found in the acidogenic phase of a two-stage digester using different combinations of carboxylic acids with two to five carbons. Exposure times up to 4 days were tested at a temperature of 35°C; however, the pH was never

below 5.6, and the best treatment only provided a 20% reduction in *Ascaris* viability (Rojas-Oropeza et al., 2016).

A paper by Butkus et al. (2011) provided the most thorough analysis of *Ascaris* egg inactivation from exposure to carboxylic acids. *A. suum* eggs were exposed to various concentrations of *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid at four different pH ranges in aqueous solutions. For all treatments, eggs were exposed to the carboxylic acids for 20 hours at 37°C. Similar to the studies cited above with bacteria, the toxicity of carboxylic acids was found to increase with carbon chain length and with increasing concentration of undissociated carboxylic acid, either through increasing the total concentration of undissociated acid plus conjugate base or by decreasing the pH.

Butkus et al. (2011) reported pH, carboxylic acid concentration, and resulting percent viability for each treatment. Using this information, I calculated the undissociated carboxylic acid concentrations and was able to examine relationships between percent viability and undissociated acid concentration (**Figure 2.3**). Based on the calculated regression equations, the concentrations of undissociated carboxylic acid required to reduce egg viability below the detection limit (0.1%) were: 581 mM *n*-butyric acid; 200 mM *n*-valeric acid; and 37.2 mM *n*-caproic acid. These results are consistent with the findings that carboxylic acid toxicity increases with carbon chain length, as discussed above. Acetic acid and propionic acid were also tested, but they were not found to be inhibitory at the concentrations tested (maximum of 1500 mM acetic acid 1320 mM propionic acid). It is important to note that these inhibitory concentrations only apply when a 20-h exposure time and temperature of 37°C are used. In comparison, a separate study by Riungu et al. (2018) reported inactivation times of

less than 4 days with a mixture of carboxylic acids at a concentration of 108 mM and temperature of 35°C. The treatments in this study used HFM mixed with different ratios of mixed food waste. The mixture likely contained mostly acetic acid, propionic acid, and *n*-butyric acid so the speed of inactivation seems faster than expected, especially if acetic acid is the dominant carboxylic acid present. There were also two treatments in this study that contained high ratios of food waste to HFM. The undissociated carboxylic acid concentrations in these treatments were lower than in other treatments, but *Ascaris* inactivation was faster, indicating that factors other than carboxylic acid concentrations affected *Ascaris* viability in this study (Riungu et al., 2018).

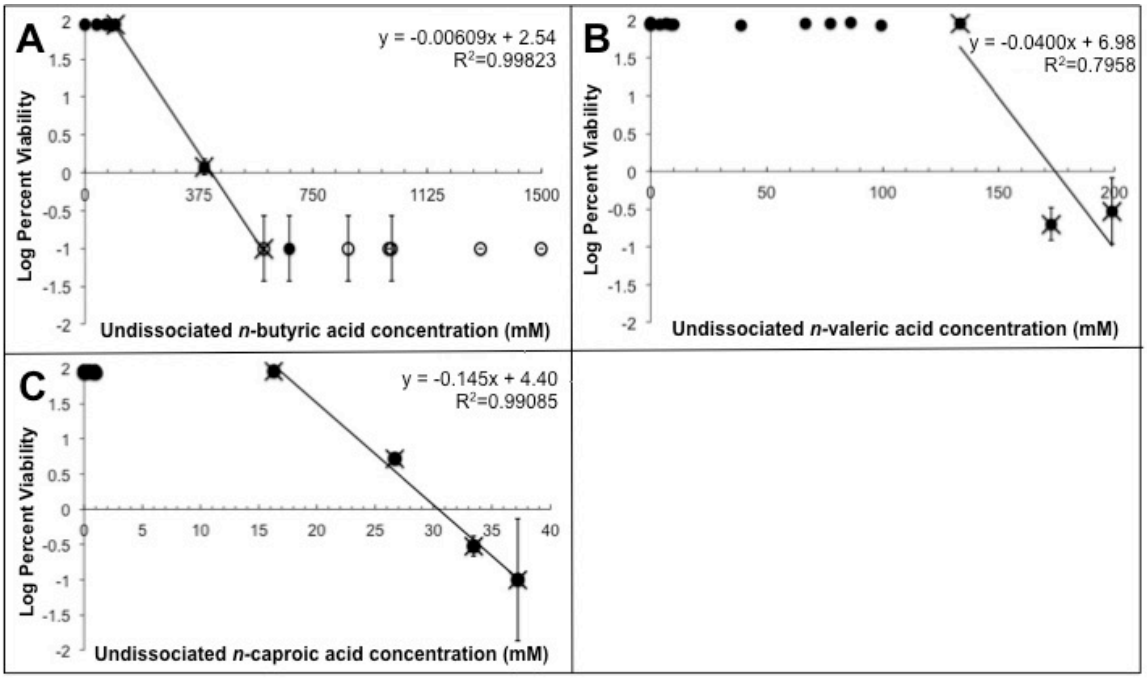

Figure 2.3 *A. suum* egg inactivation by exposure to carboxylic acids in aqueous solution for 20 h at 37°C. Data taken from Table 1 in Butkus et al. (2011). Undissociated carboxylic acid concentrations were calculated using the given total concentrations and pH values for each treatment. The pK_a values used were 4.81, 4.80, and 4.88 for *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid, respectively (Stratford and Eklund, 2003). Note that the scale of the x-axis is different for each panel. Panels (A), (B), and (C) show results for exposure to *n*-butyric, *n*-valeric, and *n*-caproic acids,

respectively. Open symbols (○) indicate points that were below the detection limit. Viability at these points was plotted as equal to the detection limit (assumed to be 0.1% viability based on 1000 eggs used for each treatment). Points with an X indicate those used to draw the linear regression curve. The linear regressions do not account for the range of concentrations that caused no decrease in viability (a lag phase), and any concentrations tested that were larger than the first one to cause complete inactivation were not included. Error bars denote the propagated standard deviation between triplicate samples.

An initial survey was also performed testing the toxicity of carboxylic acids to *A. suum* eggs suspended in anaerobic digester sludge (Butkus et al., 2011). Propionic, *n*-butyric, and *n*-valeric acids were individually tested at only one concentration. The pH was held constant at 4, and the temperature was 37°C. Exposure times of 20 hours and 7 days were tested. Interestingly, 1320 mM propionic acid reduced egg viability to 39.7% after 20 hours in digester sludge, while it did not cause any reduction when eggs were exposed at the same time and concentration in aqueous solution. After 7 days, the same concentration reduced egg viability below the detection limit. Treatments of 1170 mM *n*-butyric acid and 174 mM *n*-valeric acid for 20 hours in digester sludge also reduced egg viability below the detection limit. Based on the relationships shown in **Figure 2.3**, the result for *n*-butyric acid was not surprising. However, the relationship for *n*-valeric acid predicted that a concentration greater than 200 mM would be required to reduce viability below detection. These results for eggs suspended in sludge, particularly from propionic acid, suggest that carboxylic acids may be more effective at reducing *Ascaris* viability when eggs are exposed in sludge than when exposed in aqueous solutions (Butkus et al., 2011). Other studies have also found that exposing *Ascaris* eggs to chemical disinfectants in aqueous solutions will produce more conservative results than if eggs were exposed in sludge (Nordin et al., 2009; Pecson et al., 2007; Schuh et al.,

1985). Pecson et al. (2007) proposed that this trend is due to additional compounds, such as ammonia, other organic acids, aldehydes, and alcohols, found in sludge that may provide additional inhibitory effects to the eggs.

Recent work has shown that increased temperature also increases the toxicity of carboxylic acids to *Ascaris* eggs (Islam, 2014). *A. suum* eggs were exposed to three different carboxylic acids at different concentrations (*n*-butyric acid at 869 mM, *n*-valeric acid at 174 mM, and *n*-caproic acid at 33.4 mM). Each concentration was also tested at two different temperatures: 22°C and 37°C. The 15°C increase was found to considerably increase the inactivation ability of the carboxylic acids. For *n*-butyric acid, viability was reduced below detection after one hour for eggs exposed at 37°C, while no viability reduction was observed for eggs exposed at 22°C after 10 hours. For *n*-valeric acid and *n*-caproic acid, viability was reduced below detection after 12 hours for eggs exposed at 37°C, while eggs remained around 80% viable after 48 hours for eggs exposed at 22°C (Islam, 2014).

2.4 Production of carboxylic acids

If the toxicity of carboxylic acids to *Ascaris* eggs and other pathogens is to be utilized in a sanitation system, the carboxylic acids must be produced in a cost-effective way. This can be done through the carboxylate platform under which reactor microbiomes convert complex organic wastes to a mixture of carboxylic acid intermediates that can be utilized through multiple pathways (Agler, Spirito, et al., 2012; Agler, Werner, et al., 2012). Anaerobic digestion is one carboxylate platform process that converts the carboxylic acid intermediates to methane-rich biogas. Another pathway of particular

interest here is reverse β-oxidation. Through this process, two carbons at a time are cyclically added to the chains of short-chain carboxylic acids (e.g., acetate [C2] and *n*-butyric acid [C4]) to produce medium-chain carboxylic acids (e.g., *n*-caproic acid [C6] and *n*-caprylic acid [C8]). An electron donor, such as ethanol or lactate, is required in this pathway to provide electrons to the short-chain carboxylic acid electron acceptors (Spirito et al., 2014).

Several continuous bioreactor studies have used synthetic substrates of ethanol and acetate to demonstrate sustained production of *n*-caproic acid and *n*-caprylic acid by reactor microbiomes (Grootscholten, Steinbusch, et al., 2013a, 2013b; Kucek, Spirito, et al., 2016; Steinbusch et al., 2011). Some of these reactors have achieved the highest production rates of *n*-caproate seen in the literature (Angenent et al., 2016), with one study reporting *n*-caproic acid productivities up to 484 mM/d (55.7 g L^{-1} d^{-1}). (Grootscholten, Steinbusch, et al., 2013b). Two recent studies have also successfully produced *n*-caproic acid using synthetic substrate with lactate as the electron donor (Kucek, Nguyen, et al., 2016; Zhu et al., 2015). Ultimately, however, the carboxylate platform should utilize real organic waste streams as substrates. Some bioreactor studies have used complex organic substrates but continued to add ethanol as an exogenous electron donor (Grootscholten, dal Borgo, et al., 2013; Grootscholten et al., 2014; Kenealy et al., 1995; Liang and Wan, 2015; Weimer et al., 2015). Another series of studies have used ethanol-rich corn beer from corn ethanol production as substrate (Agler, Spirito, et al., 2012; Ge et al., 2015). Although additional ethanol was not added to the reactor, the substrate was already very rich in ethanol. Similarly, a continuous reactor using ethanol-rich wine lees as substrate was operated with high production

rates of *n*-caproate and *n*-caprylate (Kucek, Xu, et al., 2016). A recent study utilized acid whey from Greek yogurt production to produce MCCAs by first converting the lactose in the whey to lactate, which can be used in place of ethanol as an electron donor (Xu et al., 2018). This is the first study that did not require an initial high concentration of electron donor or addition of an external electron donor.

HFM consists of approximately 33% undigested carbohydrate fiber (e.g., cellulose), 33% microbial biomass, and 33% lipids and proteins (primarily oleic acid and casein) (Kaba et al., 1990; Wignarajah et al., 2006). Carboxylate platform research performed using cellulosic materials and other carbohydrates as substrates with no additional electron donor are therefore particularly interesting. As part of the carboxylate platform, reactor microbiomes break down complex organic compounds to pyruvate and then carboxylic acid intermediates and ethanol through hydrolysis and primary fermentation (Agler et al., 2011). These intermediate products can provide both the electron acceptors and electron donors to be fed into the reverse β-oxidation pathway to produce desired longer-chain carboxylic acids (Agler et al., 2011; Spirito et al., 2014). Several studies attempting to ferment carbohydrates for hydrogen production demonstrated that carbohydrate substrates with no added electron donor can be used to produce and chain elongate carboxylic acids. Wang et al. (2007) and Zhao et al. (2008) fed sucrose-rich substrate to anaerobic reactors and found significant production of ethanol, *n*-butyric acid, and *n*-caproic acid. Zhao et al. (2008) also fed a lactose-rich substrate but found that the major carboxylic acid product switched from *n*-caproic acid to *n*-butyric acid under this condition. Gómez et al. (2009, 2006) simulated the organic fraction of municipal solid waste (OFMSW) using a substrate of 10% banana, 10%

apple, 10% orange, 35% cabbage, 25% potatoes, 8% bread, and 2% paper mixed and ground to a particle size less than 3 mm. The authors observed standing concentrations up to about 35 mM *n*-caproic acid in the bioreactor (Gómez et al., 2009). A comparison of *n*-caproic acid production rates for many of the studies listed above can be found in Angenent et al. (2016).

Just as carboxylic acids are toxic to pathogens, they are also inhibitory to the communities that produce them (Angenent et al., 2016; Ge et al., 2015). Two strategies in reactor operation have been utilized to prevent product inhibition as carboxylic acids are produced. The first is an in-line membrane liquid-liquid extraction (pertraction) system that continuously removes product as it is formed (Agler, Spirito, et al., 2012; Ge et al., 2015; Kucek, Nguyen, et al., 2016). The second is to maintain the pH of the reactor near neutral, thereby keeping the carboxylic acids primarily in the conjugate base form that is not toxic (Grootscholten, Steinbusch, et al., 2013a, 2013b; Grootscholten et al., 2014; Kenealy et al., 1995; Steinbusch et al., 2011). The latter strategy requires either chemical addition or very short solid retention times to prevent acetoclastic methanogensis, which would compete with the desired chain elongation pathway. Both of these strategies allow higher total concentrations (undissociated acid plus conjugate base) of carboxylic acids to be produced. The pH can be reduced later to increase the fraction of the total concentration that is in the undissociated acid form, which is necessary for pathogen inactivation.

In one study with in-line pertraction, 7.5 mM undissociated *n*-caproic acid was found to be inhibitory for the particular microbiome of that reactor (Ge et al., 2015). Studies with neutral pH report very low concentrations of undissociated *n*-caproic acid;

therefore, product inhibition is likely not the limiting factor for these cases (Grootscholten, Steinbusch, et al., 2013a, 2013b; Grootscholten et al., 2014; Kenealy et al., 1995; Steinbusch et al., 2011). Carbohydrate-fed systems without extraction and pH maintained between 5 and 6 found higher inhibitory concentrations of 7-20 mM undissociated *n*-caproic acid (Gómez et al., 2009; Wang et al., 2007; Zhao et al., 2008).

One particularly interesting study by Tao et al. (2014) reported standing concentrations of up to 48 mM undissociated *n*-caproic acid in a functioning microbiome. The study examined the chemical composition of mud lining fermentation pits used to make Chinese Strong-Flavored Liquor. This liquor-producing process is thousands of years old, and every new pit is inoculated with the mud from an older pit, meaning the microbiome has likely been shaped to tolerate high concentrations of undissociated carboxylic acids over thousands of years. Tao et al. (2014) examined this mud in pits of four different ages (1, 10, 25, and 50 years). The one-year-old pits contained the most acidic mud (average pH of 3.6). Therefore, they also contained the highest concentrations of undissociated *n*-caproic acid (average of 47 mM) even though they had the lowest total concentrations (average of 50 mM undissociated acid plus conjugate base). The 25-year-old pits contained the highest total concentration of *n*-caproic acid (average of 172 mM), but a less acidic pH of 5.5 kept the concentration of undissociated *n*-caproic acid lower, at 33 mM. It should be noted that the reported concentrations of undissociated *n*-caproic acid are heavily dependent on the reported pH values. Since measurements were taken of a complex mud matrix, it is possible that pH values and carboxylic acid concentrations were not uniformly distributed, and samples may not be representative of the entire matrix. However, five different fermentation pits

were sampled for each age, and standard deviations for measured values were relatively low, which suggests that the reported values are representative of the entire matrix (Tao et al., 2014). While these high concentrations of undissociated *n*-caproic acid have not been seen before in carboxylate platform reactors, the study provides evidence that microbiomes may be shaped over time to develop increased tolerance to undissociated carboxylic acids. This will be critical for sanitation applications because the ideal microbiome would be more resistant to carboxylic acid toxicity than the pathogens present in the system.

2.5 Conclusion

This review has established the feasibility of a novel sanitation system based on fermentation of HFM to produce carboxylic acids. First, substrates with similar compositions as HFM have been used to produce and chain elongate carboxylic acids through the carboxylate platform in previous studies. Second, we know that chain elongation will be important due to the increased toxicity of carboxylic acids with longer carbon chains. We also know that the pH in the system must be reduced to keep a larger fraction of the total carboxylic acid concentration in the undissociated form, which is toxic to pathogens, including *Ascaris* eggs.

CHAPTER 3

INACTIVATION OF *ASCARIS* EGGS IN HUMAN FECAL MATERIAL THROUGH *IN-SITU* PRODUCTION OF CARBOXYLIC ACIDS

Adapted from: Harroff, L.A., Liotta, J.L., Bowman, D.D., Angenent L.T. 2017. Inactivation of *Ascaris* eggs in human fecal material through in situ production of carboxylic acids. *Environ. Sci. Technol., 51*(17), 9729-9738.

3.1. Abstract

Discovering new ways to inactivate pathogens in human waste is critical to improve worldwide access to sanitation and to reduce the environmental impact of conventional waste treatment processes. Here, we utilized the carboxylate platform and chain elongation to produce *n*-butyric acid and *n*-caproic acid via anaerobic fermentation of human fecal material. Then, we inactivated *Ascaris* eggs through exposure to these carboxylic acids. Using batch experiments with human fecal material as substrate, we accumulated *n*-butyric acid and *n*-caproic acid at total concentrations (uncharged acid plus conjugate base) of 257 mM and 27.1 mM, respectively. We then showed that carboxylic acids at these concentrations inactivated *Ascaris* eggs when the pH was below the pKa for the acids, causing them to exist primarily in the uncharged forms. We observed that uncharged carboxylic acids affected viability, rather than the pH itself or conjugate bases. In addition, we modeled the viability of *Ascaris* eggs as a function of uncharged carboxylic acid concentration for *n*-butyric acid and *n*-caproic acid at exposure times of 2, 6, 12, and 20 days. The results presented here indicate that *in-situ* biological

production of carboxylic acids in HFM provides a promising method of pathogen inactivation and may lead to new developments in sanitation technology and treatment of fecal sludge.

3.2 Introduction

With 2.4 billion people in the world lacking basic sanitation facilities, we face a challenge and opportunity to increase access to sanitation while also transforming the common notion of modern sanitation (UNICEF and WHO, 2015). Most developed countries rely on flushing toilets and significant investment of capital and energy to provide wastewater infrastructure and treatment. For example, in the United States, wastewater collection and treatment accounts for 3-4% of total energy use and the emission of more than 45 million tons of greenhouse gases each year (U.S. EPA, 2013). Many developing countries currently lack this infrastructure and may seek alternative sanitation solutions due to the high capital cost, difficulty of excavation for sewer lines, and poor institutional support (Larsen et al., 2016; Tilley et al., 2014). Human excreta are also rich in valuable nutrients, including nitrogen, phosphorus, and potassium, which can be recovered for reuse (Morgan and Mekonnen, 2013; Schouw et al., 2002). Therefore, new technologies are needed to safely inactivate pathogens in human waste while wasting fewer resources.

Here, we utilize the carboxylate platform and chain elongation to investigate a novel approach to inactivate pathogens in human fecal material (HFM). The carboxylate platform encompasses many processes that utilize reactor microbiomes to convert complex organic wastes to a mixture of carboxylate intermediates that can be further

upgraded to desirable products, such as methane gas in the case of anaerobic digestion or medium-chain carboxylic acids in the case of chain elongation (Agler et al., 2011). For chain elongation, bacteria first break down organic wastes into short-chain carboxylic acids (acetic, propionic, lactic, and *n*-butyric acids). If an energy-rich electron donor is available and methane production is inhibited, then the electron donor can be oxidized to acetyl-CoA, which is used to chain elongate the carboxylic acids two carbons at a time via the reverse β-oxidation pathway, as described by Spirito et al (2014). Through this process, acetic acid (C2) can be chain elongated to *n*-butyric acid (C4), and *n*-butyric acid can be chain elongated to *n*-caproic acid (C6); further chain elongation to C8 is also possible (Kucek, Spirito, et al., 2016; Spirito et al., 2014). Existing studies have demonstrated chain elongation from complex substrates, such as the organic fraction of municipal solid waste (Grootscholten, dal Borgo, et al., 2013; Grootscholten et al., 2014), fermentation beer from corn-to-ethanol production (Agler et al., 2014; Ge et al., 2015), and wine lees, which is a waste stream from wine production (Kucek, Xu, et al., 2016). However, these studies either required addition of an electron donor, such as ethanol, or contained naturally high ethanol concentrations. Here, we demonstrated the first use of human fecal material (HFM) with no additional electron donor as substrate for chain elongation.

Previous studies have shown that carboxylic acids inhibit growth of many bacteria (Abdul and Lloyd, 1985; Kunte et al., 2000, 2004; Liu et al., 2013; Royce et al., 2013; Salsali et al., 2006; Skřivanová et al., 2006), and recent work has also investigated their toxicity to *Ascaris* eggs (Barrios et al., 2007; Butkus et al., 2011; Rojas-Oropeza et al., 2016). In HFM, *Ascaris lumbricoides* eggs are considered the most resistant pathogens to

waste treatment processes due to the hydrophobicity of the innermost lipid layer of the egg (Arfaa, 1978; Bowman et al., 2000; Feachem et al., 1983; U.S. EPA, 2003). At a pH below the pK_a (~4.8), carboxylic acids exist primarily in the uncharged acid form, which is more hydrophobic than the corresponding conjugate base and able to cross the lipid layer. Butkus et al. (2011) exposed *Ascaris* eggs to solutions of carboxylic acids for 20 h at 37°C at different pH values and concluded that carboxylic acids were more effective when the pH was below the pK_a of the acid and that carboxylic acids with longer carbon chains are more effective, likely due to the increased hydrophobicity of the chain (2011). However, this study only predicted combinations of concentration and pH that were effective at one exposure time (20 h). The examined concentrations were also relatively high, particularly for *n*-butyric acid (1 M), and it would be inefficient and expensive to supply commercially available carboxylic acids to inactivate pathogens in HFM at those concentrations. Ammonia has also been extensively studied as a means of inactivating *Ascaris* eggs in HFM due to the ability of uncharged NH_3 to cross the lipid layer of the egg (Fidjeland et al., 2015; Fidjeland et al., 2016; Nordin et al., 2009; Pecson et al., 2007). While carboxylic acid inactivation requires a pH below the pK_a of the acid, ammonia inactivation requires a pH above the pK_a (9.3 at 25°C) (Pecson and Nelson, 2005). Because additional ammonia cannot be produced within fecal material, ammonia inactivation usually requires addition of external urea or ammonia solution to provide sufficient NH_3 concentrations and to increase the pH, which can be cost prohibitive (Fidjeland et al., 2016).

Therefore, we propose to inactivate pathogens in HFM by promoting *in-situ* biological production and chain elongation of carboxylic acids. Specifically, we

performed a batch fermentation experiment to test the feasibility of using HFM as substrate for chain elongation. We then investigated parameters that affect the toxicity of carboxylic acids to *Ascaris* eggs, including the acid species (uncharged versus charged), concentration, length of carbon chain, and exposure time. We focused on *n*-butyric acid and *n*-caproic acid due to the preferential production of even-chained carboxylic acids from chain elongation and the need for longer carbon chains to inactivate *Ascaris* eggs. We emphasized inactivating *Ascaris* eggs using concentrations of carboxylic acids that were feasible to be produced within HFM, and we developed relationships between required concentrations and exposure times.

3.3 Materials and methods

3.3.1 Experiment 1: Batch fermentation of HFM to produce n-butyric acid and n-caproic acid

Batch fermentations were carried out in four 1-L glass bioreactors with 3.8-cm diameter side ports for sample collection. The side ports and the tops of the bioreactors were both sealed with rubber stoppers. A plastic airlock was inserted through the top stopper to prevent pressure buildup from gas production, while also preventing oxygen from entering the bioreactor. Each bioreactor was filled with 205 (+/- 2.5) g of HFM (wet weight) and 20 g of inoculum. HFM was obtained from anonymous volunteers who used a designated restroom and placed fresh stool samples in a cooler. The samples were stored for no more than 9 h and then transferred to a -20°C freezer for longer-term storage. Once sufficient material was collected, the samples were thawed at room temperature, combined, and thoroughly mixed. The inoculum was derived from a well-

characterized reactor microbiome that was fed ethanol-rich yeast fermentation beer semi-continuously and that produced *n*-caproic acid for approximately four years (Agler, Spirito, et al., 2012; Ge et al., 2015). The inoculum was triple-washed to remove residual substrate, as described previously (Kucek, Nguyen, et al., 2016). The headspace of each bioreactor was sparged with nitrogen gas for one min before sealing the bioreactor. Bioreactors were incubated at 30°C in the dark for 78 days.

Samples were collected from three replicate bioreactors every four days for the first 20 days of fermentation and then on days 25, 32, 39, 46, 69, and 78. The sampling frequency was increased after 20 days to reduce the change in headspace and content volume over the course of the experiment. At each sampling time, the bioreactor was opened on the lab bench, the content was manually mixed with a metal spatula, and 3.5 (+/- 0.5) g of material was collected. The headspace was then sparged with nitrogen gas for one min, and the bioreactor was resealed. A fourth bioreactor was only sampled on day 0 and at the end of the experiment on day 78. The results from this bioreactor were compared to the end-point samples from the other three bioreactors to determine whether disturbances caused by sampling (e.g., periodic exposure to oxygen, manual mixing, and decreasing volume of material in the bioreactors over time) affected the production of carboxylic acids. Gas samples were also collected from the fourth bioreactor throughout the fermentation period to measure the headspace gas composition. Gas samples of 500 µl were collected through a septum that was fitted in the top rubber stopper of the bioreactor. A gas-tight glass syringe was used to collect the samples and to directly inject them into a gas chromatograph for analysis. Gas samples were not collected from the

other three bioreactors because the headspace was frequently disturbed during solids sampling.

3.3.2 Experiment 2: Effect of pH and carboxylic acid species on inactivation of Ascaris eggs

Eggs of the pig roundworm *Ascaris suum* were used as surrogates for *A. lumbricoides* eggs due to the similarities in structure and the reduced risk of infection to humans (Feachem et al., 1983). *A. suum* eggs were collected from fecal material in the intestines of naturally infected slaughterhouse pigs. To remove large debris, the fecal material was passed through a series of six sieves ranging from US 10-US 100 before final collection on a US 500 sieve (Collick et al., 2007; Kato et al., 2003). The collected eggs were further separated from small fecal material by centrifugal flotation at 800 x g for 5 min with magnesium sulfate solution (specific gravity = 1.2). Eggs were stored at 4°C in a 0.1 N H_2SO_4 solution to prevent mold growth.

The first objective in understanding *Ascaris* egg inactivation by carboxylic acids was to isolate the effect of the concentration of the uncharged acid species from the possible effects of pH and concentration of the conjugate base. We tested the same concentration of uncharged carboxylic acids at three different pH values. A combination of *n*-butyric acid and *n*-caproic acid was used at a constant 10:1 ratio, based on the results obtained from Experiment 1. The same five concentrations of uncharged acids were tested at each of the pH values (2, 4, and 5) (**Table A1.1**). The Henderson-Hasselbalch equation (**Equation 3.1**) was rearranged (**Equation 3.2**) to determine the total concentration of the uncharged acid plus conjugate base ($[HA]+[A^-]$) required to achieve

equal concentrations of the uncharged acid ([HA]) at each pH (Clugston and Flemming, 2000):

$$pH = pK_a + log\frac{[A^-]}{[HA]} \qquad \textbf{(Equation 3.1)}$$

$$[HA] + [A^-] = [HA]\left(10^{(pH-pKa)} + 1\right) \qquad \textbf{(Equation 3.2)}$$

The pK_a is 4.82 for *n*-butyric acid and 4.88 for *n*-caproic acid at 25°C, and the pK_a values of carboxylic acids vary only slightly at moderate temperatures (Perrin et al., 1981). Solutions for each treatment were created using the calculated ([HA] + $[A^-]$) concentrations for *n*-butyric acid and *n*-caproic acid (**Table A1.1**), and the pH was adjusted using 5 M HCl and 5 M NaOH.

For each exposure, approximately 1,000 *A. suum* eggs were suspended in a microcentrifuge tube in 1.0 mL of the corresponding carboxylic acid solution. Each treatment was tested in triplicate. A control condition in which *A. suum* eggs were suspended in deionized (DI) water with no acid and no pH adjustment was also tested in triplicate. Eggs were exposed to the acid solutions for three days while incubated at 37°C in a heating block on the bench. After the exposure period, each tube was centrifuged at 3,500 x g for 4.5 min. The supernatant was collected to verify pH and carboxylic acid concentrations, and the eggs were resuspended in a sodium phosphate buffer solution (10 mM, pH 7.0) to neutralize the acids. The centrifugation and resuspension process was repeated two additional times to thoroughly remove the carboxylic acids. The eggs were then transferred to a 24-well plate, and 1.0 mL of 0.1 N H_2SO_4 was added to each well to prevent mold growth, as recommended by the standard EPA method for enumerating viable eggs (U.S. EPA, 2003). The eggs were incubated at 28°C for three weeks in the dark. After incubation the outer corticated egg layer was removed by adding sodium

hypochlorite (final concentration: 0.6% w/v), and the eggs were examined microscopically to determine viability (Butkus et al., 2011; Collick et al., 2007). Eggs that had formed larvae were considered viable; all others were counted as nonviable (Kato et al., 2003; Nordin et al., 2009; Pecson et al., 2007). Five hundred eggs were examined in each well. If no viable eggs were found in the initial 500, the remaining eggs in the well (to a maximum of 1,000) were examined. We calculated the fraction viable by: 1) dividing the number of viable eggs with the total number of counted eggs; and 2) normalizing to the mean viability of the control eggs. The average viability of all control eggs was 88.8% (SD=3.6%). For all viability calculations, we did not determine percent recovery of eggs. If some eggs were destroyed, our estimates of inactivation would be conservative.

3.3.3 Experiment 3: Effect of carboxylic acid concentration, carbon chain length, and exposure time on inactivation of Ascaris eggs

The individual effects of *n*-butyric acid and *n*-caproic acid on *A. suum* viability across a range of exposure times were also tested. Four different exposure times (2, 6, 12, and 20 days) with eight different concentrations of *n*-butyric acid and *n*-caproic acid (individually) were tested at a pH of 2.0 to maintain > 99% of the acids in the uncharged form (**Table A1.2**). Duplicate samples were included for two of the eight concentrations at each exposure time to verify reproducibility. For the remaining six concentrations, only one sample was tested. Controls were tested in triplicate for each exposure time using DI water with no acids added and no pH adjustment.

Approximately 1,000 *A. suum* eggs were suspended in a microcentrifuge tube in 1.0 mL solution of either *n*-butyric acid or *n*-caproic acid. Eggs were incubated in the dark at 30°C in a sealed gas-tight chamber that was made anaerobic using GasPak EZ Anaerobe Sachets (BD, Sparks, MD). Microcentrifuge tubes were left open for gas exchange and covered with Parafilm® (Bemis NA; Neenah, WI) to prevent evaporation. A lower temperature (30°C) was used for this experiment because thermal inactivation can start to occur at 37°C during the longer incubation times that were used here, which would confound conclusions regarding carboxylic acids (Kato et al., 2003; Manser et al., 2015). Anaerobic conditions were maintained to simulate conditions required to produce the carboxylic acids and to keep all eggs in the single-cell stage throughout the exposure period, as the egg shell is believed to weaken during larval development (Manser et al., 2015). After the designated exposure period, eggs were washed and transferred to a 24-well plate as described above. The eggs were examined microscopically to confirm that no larvae had developed during the exposure period, and they were incubated in the dark for three weeks at 28°C. Eggs were examined, and fraction viable was calculated as described above. Samples of the acid solutions were collected before and after the exposure periods to confirm carboxylic acid concentration.

3.3.4 Experiment 4: Inactivation of Ascaris eggs by n-butyric acid and n-caproic acid in a HFM matrix

The effect of exposing *Ascaris* eggs to a combination of *n*-butyric acid and *n*-caproic acid within a HFM matrix was tested to simulate more realistic conditions for a sanitation system. Five treatments were used to include proper controls: (1) spiked acids and low

pH in HFM; (2) no added acids and low pH in HFM; (3) spiked acids and natural pH in HFM; (4) no added acids and natural pH in HFM; and (5) no added acids and natural pH in autoclaved HFM (**Table A1.3**). Each treatment was tested in triplicate. For Treatments 1 and 3 with spiked acids, *n*-butyric acid and *n*-caproic acid were added to HFM to achieve final concentrations of 260 mM and 26 mM, respectively. These concentrations were chosen based on results from Experiment 1 to simulate the carboxylic acid conditions that can be produced biologically from fermentation of HFM. Acids were spiked into unfermented fecal material for these treatments to better isolate the inactivation effect of carboxylic acids from the potential effects of other substances. For Treatments 1 and 2, the pH was adjusted to a target of 4.5-5.0 using 12 M HCl. For Treatment 3, the pH was raised using 5 M NaOH after adding the carboxylic acids to equal the unadjusted pH of Treatment 4 (pH=6.40). DI water was used to balance the total volume of liquid added to each treatment (from pH adjustment and carboxylic acid addition) to maintain equal solids concentrations. For Treatment 5, the HFM was autoclaved at 120°C and 100 kPa for a 30-min liquid cycle to prevent any biological activity from occurring during the exposure period that may affect *Ascaris* viability. For each treatment, 7 g of prepared HFM (wet weight) and approximately 5,000 *A. suum eggs* were placed in a 15-mL glass serum bottle. Serum bottles were sealed, mixed by vortexing, and the headspace was sparged with nitrogen gas. The septum of each bottle was punctured with a 21-G needle connected to an airlock to prevent pressure buildup from gas production while keeping the bottle anaerobic. Bottles were incubated at 30°C for exposure times of 5, 15, and 19 days. Three replicates were used for each combination of treatment and exposure time. After the exposure period, 1 g of contents was removed

to measure pH and carboxylic acid concentrations. The remaining contents were rinsed from the serum bottle into a 50-mL conical tube with DI water. The tube was centrifuged at 800 x g for five min to pellet the *A. suum* eggs. The supernatant was discarded, and the pellet was suspended in a sodium phosphate buffer solution (10 mM, pH 7.0) and centrifuged again at 800 x g for five min. *A. suum* eggs were separated from the remaining sediment through centrifugal flotation (as described above), sieving through US 100 and US 200 sieves, and then collecting eggs on a US 500 sieve. The collected eggs were suspended in 0.1 N H_2SO_4 and transferred to a 24-well plate. Each well was examined microscopically to determine that *A. suum* eggs had been successfully recovered and that they remained in the single-cell stage throughout the exposure time. The eggs were then incubated at 28°C for three weeks in the dark, and 250 eggs from each replicate were examined microscopically to determine viability.

3.3.5 Sample Analysis

The pH of samples was measured using a Thermo-Scientific Orion pH electrode (Waltham, MA). Carboxylic acid concentrations were measured using a gas chromatography (GC) system (Hewlett-Packard 5890 Series II; Wilmington, DE) described previously (Usack and Angenent, 2015). Briefly, a Nukol fused silica capillary column (15 m × 0.53 m × 0.50 µm; Supelco Inc., Bellefonte, PA) connected to a flame ionization detector (FID) was used with inlet and detector temperatures of 200°C and 275°C, respectively. Helium was the carrier gas, and the initial oven temperature was 70°C followed by a ramp of 12°C min^{-1} to 200°C, where it was maintained for 2 min. Headspace gas composition (carbon dioxide, methane, and nitrogen) was measured using

a GC (SRI 8610C; SRI Instruments, Torrance, CA) equipped with a thermal conductivity detector (TCD) and a packed column (0.3-m HaySep-D packed Teflon; Restek, Bellefonte, PA). Helium was used as the carrier gas, and an isothermal temperature program was used with the detector at 105°C and oven at 40°C, as described previously (Usack and Angenent, 2015).

3.3.6 Statistics

Inactivation data from Experiments 2 and 3 were fit to a two-parameter logistic model (**Equation 3.3**), where x is the concentration of uncharged carboxylic acid and a and b are empirical constants. The parameter a indicates the rate of decline in viability, and b indicates the concentration at which the fraction viable is 0.50.

$$\text{Fraction Viable} = \frac{1}{1+e^{(a(x-b))}} \qquad \textbf{(Equation 3.3)}$$

JMP (SAS Institute, Inc., Cary, NC) and R (R Foundation for Statistical Computing, Vienna, Austria) were used to fit the data and to complete statistical analyses in all experiments.

3.4 Results and discussion

3.4.1 Experiment 1: HFM can be used as a substrate for chain elongation to produce n-*butyric acid and* n-*caproic acid*

Average total concentrations (uncharged acid plus conjugate base) of *n*-butyric acid and *n*-caproic acid reached maximum values of 257 mM and 27.1 mM, respectively, after 46 days of fermentation (**Figure 3.1**). On the final sampling day (day 78), the total concentrations of *n*-butyric acid and *n*-caproic acid in the otherwise unsampled bioreactor

were 212 mM and 17.7 mM, respectively. The concentration of *n*-butyric acid was within one standard deviation of the average of the other three bottles on the same day (average=227 mM, SD=23.7 mM); however, the concentration of *n*-caproic acid was below this range (average=22.6 mM, SD=1.03 mM). This result indicated that sampling disturbances may have slightly improved performance of the bioreactors, likely due to the mixing that took place at each sampling point for the experimental bioreactors but not for the control. Therefore, mixing may be an important parameter to consider for future work. The average pH in the bioreactors initially decreased from 6.7 to 5.1 during the first four days of fermentation but then steadily increased back to 6.7 throughout the remainder of the experiment. Other carboxylic acids including acetic acid, propionic acid, *n*-valeric acid, and *i*-valeric acid were also observed in the bioreactors (**Figure A1.1A and A1.1B**).

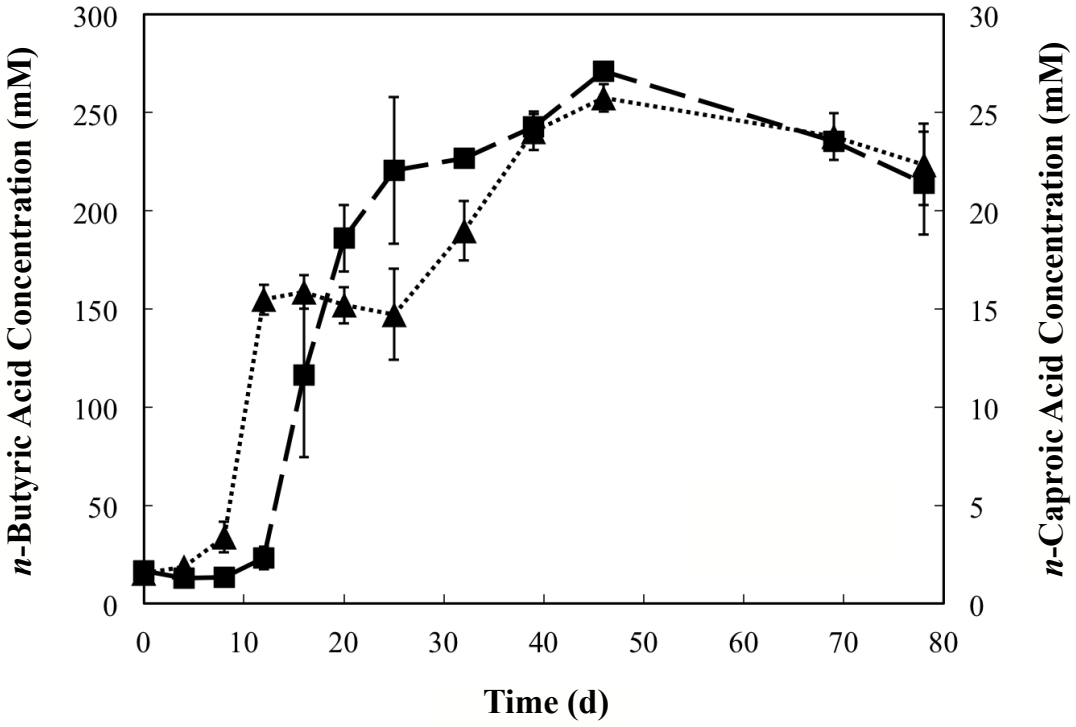

Figure 3.1 Accumulation of *n*-butyric acid and *n*-caproic acid from batch fermentation of HFM at 30°C. Concentration of *n*-butyric acid (▲) is plotted against the left y-axis, and concentration of *n*-caproic acid (■) is plotted against the right y-axis. Concentrations represent the total concentration of uncharged acid plus conjugate base. Error bars show the standard deviations of three biological replicates.

This experiment showed that HFM alone can be used as substrate to produce *n*-butyric acid and *n*-caproic acid. Although no electron donor, such as ethanol or lactate, was directly added to the bioreactors in this experiment, ethanol and lactate may have been produced as intermediate fermentation products and then used as electron donors in the chain elongation process to produce *n*-caproic acid and some of the *n*-butyric acid (Angenent et al., 2016). In addition to presence of an electron donor, inhibition of acetoclastic methanogenesis has been cited as an important criterion for chain elongation because methanogenesis can compete with chain elongation for substrate (Angenent et al., 2016; Grootscholten, dal Borgo, et al., 2013). In other studies, methanogens have

been suppressed through maintenance of a mildly acidic pH (Agler et al., 2014; Ge et al., 2015; Kucek, Nguyen, et al., 2016), short hydraulic retention times (Grootscholten, Steinbusch, et al., 2013a, 2013b), and chemical methanogen inhibitors (Steinbusch et al., 2011). Here, we employed none of these methods; however, methane concentrations in the headspace remained below our detection limit (< 0.12%) throughout the study. The lack of methane may be attributed to a lack of methanogens, both in the inoculum and intrinsically in the HFM, as well as a high organic loading rate in the bioreactors since the HFM was not diluted with water or urine.

3.4.2 Experiment 2: Concentration of the uncharged acid species drives Ascaris *inactivation*

A two-parameter logistical model was fit to the experimental data with all pH levels combined (**Equation 3.4**), where *a* and *b* from **Equation 3.3** were calculated to equal 0.0975 (standard error=0.00864, p=1.94 x 10^{-14}) and 148 (standard error=0.549, p=2.00 x 10^{-16}), respectively.

$$\text{Fraction Viable} = \frac{1}{1+e^{(0.0975(x-148))}} \qquad \textbf{(Equation 3.4)}$$

The model was a good fit with mean square error (MSE) of 1.35 x 10^{-3} and mean absolute error (MAE) of 0.0207 (**Figure 3.2**). The same model was also fit to the data with *a* and *b* calculated individually for each pH level (**Table A1.4**), but this model was not statistically different (p=0.0748) from the combined model, indicating that pH, total concentration of uncharged acid plus conjugate base, and concentration of conjugate base alone were not predictive of *Ascaris* egg viability. Thus, solely the uncharged concentration of *n*-butyric acid and *n*-caproic acid was predictive. High viability (94.9%-

95.8%) at the lowest uncharged acid concentration (110 mM) for all pH values also showed that a low pH alone did not reduce egg viability at the time and temperature tested (**Table A1.5**).

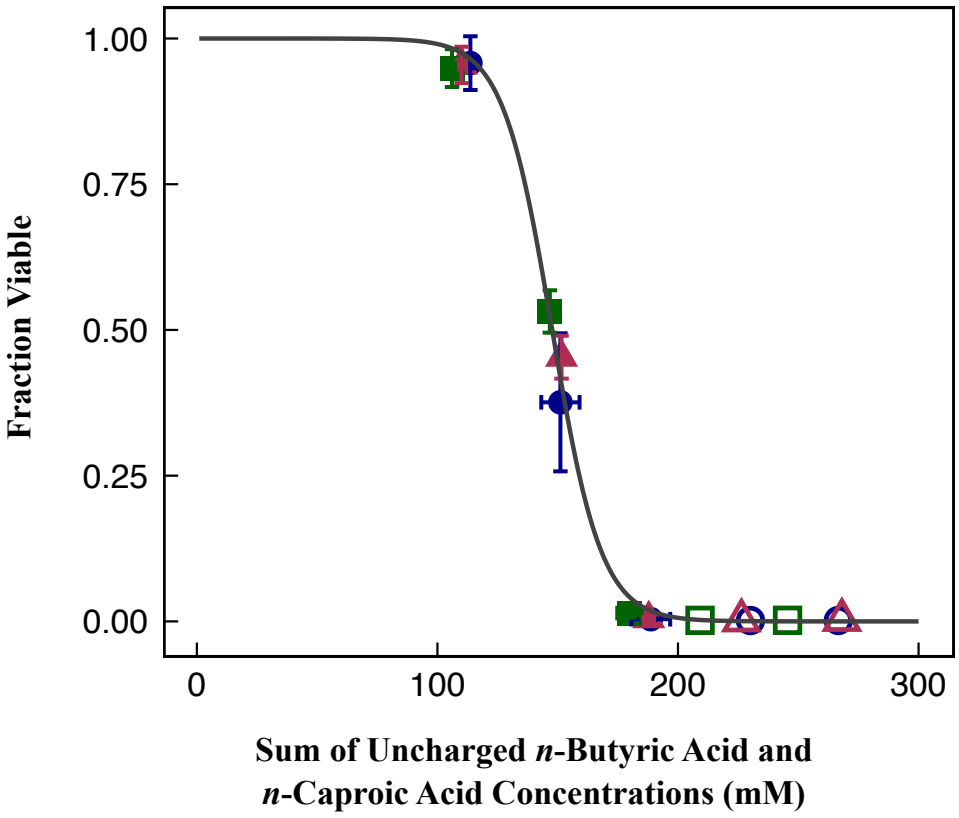

Figure 3.2 *Ascaris* egg inactivation as a function of uncharged carboxylic acid concentration. *A. suum* eggs were exposed to the same uncharged carboxylic acid concentrations at pH 2 (●), pH 4 (▲), and pH 5 (■). The solid line represents the model fitted to the data for all pH levels combined. Eggs were exposed to carboxylic acids at 37°C for three days. Open symbols indicate viability below the detection limit (average detection limit=0.00152, calculated individually for each treatment). Y-error bars represent the standard deviations of viability for three biological replicates, and x-error bars represent the standard deviations of carboxylic acid concentration as measured with gas chromatography.

The results indicate that pH only plays an indirect role in the inactivation of *Ascaris* eggs by controlling the fraction of the carboxylic acid that is in the uncharged acid form and that the concentration of conjugate base also did not affect inactivation rates at the concentrations tested. In the Butkus et al. (2011) study, toxic concentrations

of carboxylic acids (uncharged acid plus conjugate base) were modeled as functions of pH for one exposure time and temperature. Based on the results presented here, we propose that the relationship between total concentration and pH is defined only by the Henderson-Hasselbalch relationship when other parameters are held constant. This result is similar to that observed from ammonia inactivation of *Ascaris* eggs (Pecson and Nelson, 2005). Pecson and Nelson (2005) observed that pH alone did not cause egg inactivation, but pH did control the fraction of ammonia that was in the uncharged form, and the concentration of the uncharged form controlled inactivation rates. The proposed mechanism for inactivation by carboxylic acids is similar to the mechanism of ammonia. In both cases, the uncharged form of the chemical is able to cross the lipid membrane of the egg while the ionic form is not (Brul and Coote, 1999; Warren, 1962). Once inside the neutral pH environment of the *Ascaris* egg, a new equilibrium is established between the uncharged and charged species. For ammonia, excess protons in the cell are taken up to form the ammonium ion. For carboxylic acids, protons are released to form the conjugate base. The excess protons may disrupt the internal pH of the cell and the proton gradient, causing inactivation (Herrero et al., 1985; Nicolaou et al., 2010). In addition, the accumulation of conjugate base in the cell may cause increased osmolarity and lethal turgor pressure (Carpenter and Broadbent, 2009). These mechanisms have been proposed for bacteria in the past. The results presented here indicate that the same mechanisms may apply to *Ascaris* eggs.

3.4.3 Experiment 3: Ascaris *inactivation rates vary with carbon chain length and exposure time*

Longer exposure times and increased carbon chain lengths of carboxylic acids caused greater inactivation of *Ascaris* eggs (**Figure 3.3, Figure A1.2, Table A1.6**). For each combination of exposure time and type of acid, the data could be fit to the same two-parameter logistic model used previously (**Equation 3.3, Table 3.1**). The parameters *a* and *b* were statistically significant at a significance level of 0.001 for most combinations (**Table 3.1**). The exceptions were parameter *a* for six days of exposure to *n*-butyric acid and parameter *a* for 12 days of exposure to *n*-caproic acid (**Table 3.1**). For six days of exposure to *n*-butyric acid, there is an outlying point at a concentration of 336 mM *n*-butyric acid and 50% viability (**Figure 3.3A**). There were no replicates at this concentration and no known error in the experimental setup so it was not excluded from the model. Including the point reduced the significance of parameter *a*, but a comparison of the estimates for parameters *a* and *b* with and without the data point did not show a large difference. Parameter *a* was estimated to equal 0.0448 with the point included versus 0.0480 with the point excluded, and parameter *b* was estimated to equal 216 in both cases. For 12 days of exposure to *n*-caproic acid, the highest concentration tested (16.8 mM) only reduced the viability to 11%. Testing higher concentrations at this exposure time to achieve greater inactivation would have improved the accuracy and significance of the model.

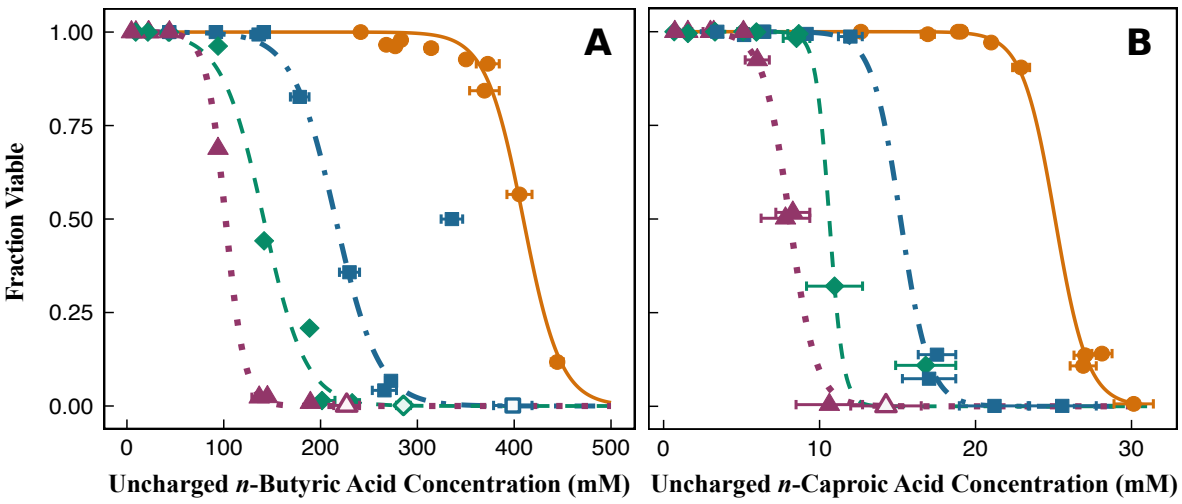

Figure 3.3 Inactivation of *Ascaris* eggs due to exposure to *n*-butyric acid (A) and *n*-caproic acid (B). *Ascaris* eggs were exposed to carboxylic acids at 30°C for four different exposure times: 2 days (●), 6 days (■), 12 days (♦), and 20 days (▲). Open symbols indicate viability below the detection limit (average detection limit=0.00127, calculated individually for each treatment). The carboxylic acid concentration shown is the average of measurements made before and after the exposure period for each treatment, and the x-error bar shows the range between the two measurements. Differences in concentration were due to volatilization of the acids over time. Replicate treatments are shown as individual points.

Table 3.1 Model parameters and statistics for inactivation of *Ascaris* eggs when exposed to *n*-butyric acid and *n*-caproic acid for 2, 6, 12, and 20 days at 30°C.

Acid	Exposure Time (d)	Parameter *a* (SE*)	*p*-value *a*	Parameter *b* (SE*)	*p*-value *b*	Mean Square Error	Mean Absolute Error
n-Butyric	2	0.0519 (4.80×10^{-3})	4.71×10^{-6}**	409 (2.23)	8.75×10^{-16} **	9.81×10^{-4}	2.82×10^{-2}
	6	0.0448 (2.03×10^{-2})	5.82×10^{-2}	216 (14.1)	3.23×10^{-7} **	2.5×10^{-2}	6.41×10^{-2}
	12	0.0462 (7.07×10^{-3})	1.82×10^{-4} **	141 (3.85)	3.36×10^{-10} **	2.07×10^{-3}	3.03×10^{-2}
	20	0.101 (4.18×10^{-3})	9.26×10^{-9} **	102 (0.388)	$< 2 \times 10^{-16}$ **	2.58×10^{-5}	3.15×10^{-3}
n-Caproic	2	0.954 (9.27×10^{-2})	6.87×10^{-6}**	25.1 (0.200)	1.83×10^{-14} **	9.87×10^{-4}	1.71×10^{-2}
	6	1.09 (0.199)	5.85×10^{-4} **	15.2 (0.377)	1.58×10^{-10} **	6.76×10^{-4}	1.42×10^{-2}
	12	2.24 (1.11)	7.84×10^{-2}	10.6 (0.179)	7.22×10^{-12} **	1.21×10^{-3}	1.30×10^{-2}
	20	1.17 (0.188)	2.54×10^{-4} **	8.08 (0.103)	7.61×10^{-13} **	1.46×10^{-3}	2.40×10^{-2}

***** SE= standard error, ****** Significant at α=0.001

For *n*-butyric acid and *n*-caproic acid, the inactivation curves shifted left with increasing exposure time, indicating that a smaller concentration of uncharged carboxylic acid was required to achieve the same level of inactivation (**Figure 3.3A and 3.3B**). This shift can be visualized by plotting the parameter *b* against exposure time for each acid (**Figure A1.3**). Using the logs of parameter *b* and exposure time, we can predict *b* as a function of time using linear regression (**Figure 3.4A and 3.4B**). More data points are needed to obtain a more accurate model, but this is the first data to correlate carboxylic acid inactivation in *Ascaris* eggs to exposure time. The parameter *a* was also examined as a function of exposure time, but no clear correlation was found (**Figure A1.4**). In addition, at the same exposure times, the required concentrations for inactivation decreased by more than an order of magnitude when using *n*-caproic acid (C6) (**Figure**

3.3B) compared to *n*-butyric acid (C4) (**Figure 3.3A**). This result supports the finding that longer chain carboxylic acids are more effective at inactivating *Ascaris* eggs (Butkus et al., 2011; Takeyama, 1951), likely due to the increasing hydrophobicity of longer chain molecules. Similar findings have also been reported for *Escherichia coli* (Abdul and Lloyd, 1985; Royce et al., 2013) and *Saccharomyces cerevisiae* (Liu et al., 2013).

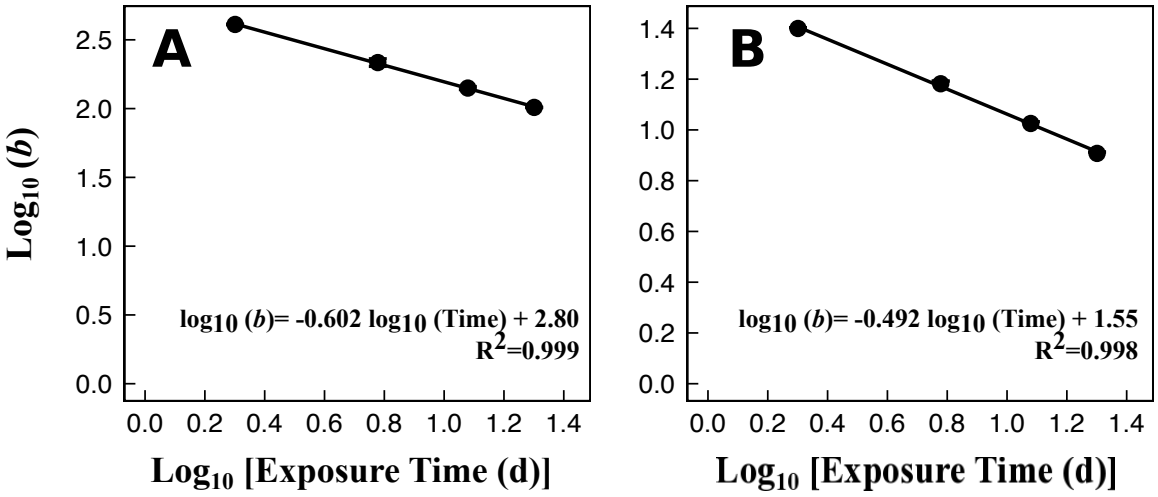

Figure 3.4 Relationship between the log of parameter *b* and log of exposure time for *n*-butyric acid (A) and *n*-caproic acid (B).

Inverse calculations using the fitted models (**Table 3.1**) were also used to estimate the concentration of each acid required to achieve > 99.9% inactivation of *Ascaris* eggs at exposure times of 2, 6, 12, and 20 days (**Figure 3.5**). We can combine this information with the concentrations that were accumulated from fermentation of HFM (**Figure 3.1**) to predict that biologically produced concentrations of *n*-butyric acid (257 mM) can inactivate *Ascaris* eggs with an exposure time between 12 and 20 days (**Figure 3.5A**). Likewise, biologically produced concentrations of *n*-caproic acid (27.1 mM) can inactivate *Ascaris* eggs with an exposure time between 2 and 6 days (**Figure 3.5B**). However, to achieve this result, the produced carboxylic acids must be in their uncharged

forms, which can be accomplished by decreasing the pH in the bioreactors below the pK_a of the carboxylic acids (4.82 for *n*-butyric acid and 4.88 for *n*-caproic acid). Comparatively, the experimental data from Butkus et al. (2011) required a minimum concentration of 398 mM uncharged *n*-butyric acid (reported as 1 M total *n*-butyric acid at pH 5.0) or 37.2 mM uncharged *n*-caproic acid (reported as 37.8 mM total *n*-caproic acid at pH 3.1) to reduce *Ascaris* viability to 0.1% or less. However, Butkus et al. (2011) only tested different concentrations at one exposure time of 0.83 d (20 h), demonstrating the importance of considering both exposure time and concentration. Using longer exposure times, we were able to reduce *Ascaris* viability below 0.1% using concentrations that could feasibly be produced from HFM in a sanitation system.

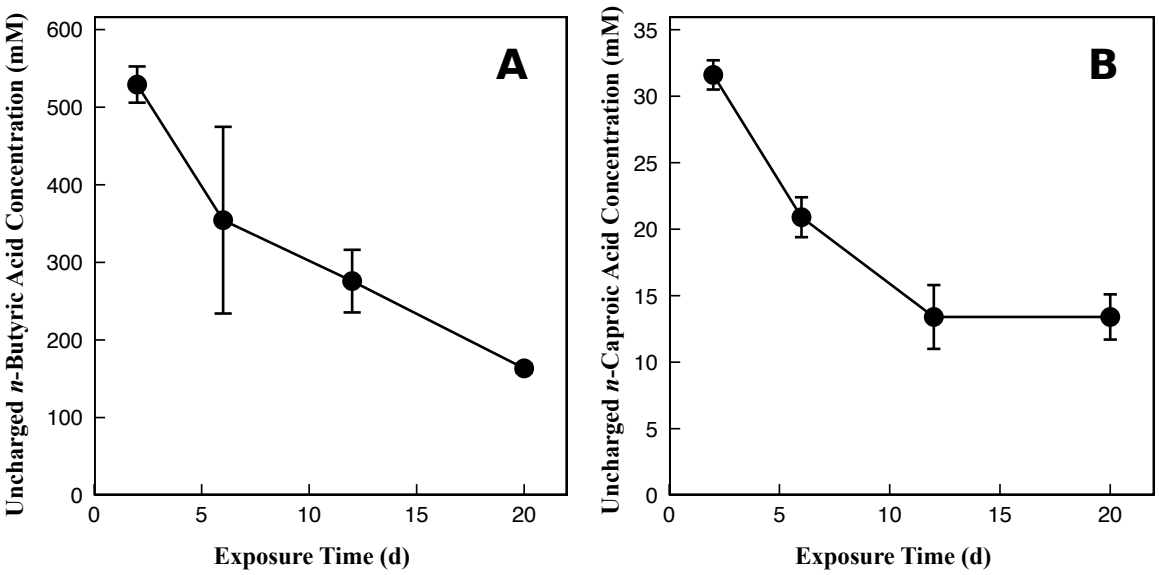

Figure 3.5 Carboxylic acid concentration and exposure time combinations to achieve > 99.9% inactivation of *Ascaris* eggs. Carboxylic acid concentrations were calculated using the fitted models (Table 3.1) with the fraction of viable *Ascaris* eggs equal to the experimental detection limit (0.001). Panel A shows concentration and time combinations for *n*-butyric acid, and panel B shows combinations for *n*-caproic acid. Error bars represent 95% confidence intervals.

3.4.4 Experiment 4: Multiple variables affect Ascaris *inactivation in a matrix of HFM*

Differences in *Ascaris* viability between Treatments 1-5 became more evident at longer exposure times (**Figure A1.5**), and the results from 19 days of exposure are discussed here. The *A. suum* eggs used in this experiment were 83% viable before treatment, and the viability of eggs from Treatments 2 and 5 (80% and 84%, respectively) did not decrease after 19 days of exposure (**Figure 3.6**). This result was expected because carboxylic acids were not added to either of those treatments. In Treatment 1 we spiked *n*-butyric acid at a concentration of 240 mM, *n*-caproic acid at a concentration of 27 mM, operated with a pH below the pK_a of both acids, and the *Ascaris* egg viability was reduced to 8.5% (**Figure 3.6**). Based on the results from Experiment 3, we anticipated that 240 mM uncharged *n*-butyric acid or 27 mM uncharged *n*-caproic acid would individually cause 99.9% inactivation of *Ascaris* eggs with a 12-20 day exposure time (**Figure 3.5**). In Experiment 4, the carboxylic acids were combined, so we expected the required exposure time to be even shorter. However, after a 19-day exposure period, the eggs remained 8.5% viable. This result may be partly explained by the pH in Treatment 1, which was 4.72 at the beginning of the exposure period and increased to 4.80 over the 19 days. With the pH so close to the pK_a values of the carboxylic acids, the average concentrations of uncharged *n*-butyric acid and *n*-caproic acid were only 132 mM and 12.4 mM, respectively (**Figure A1.6**). Based on the models from Experiment 3, we would expect those concentrations individually to require an exposure time greater than 20 days, while we only exposed for 19 days. Previous work has also suggested that protective agents in HFM may shield pathogens from inhibitory chemicals, including

carboxylic acids, and this challenge would need to be considered to improve treatment efficiency (Popat et al., 2010).

Treatment 3 also caused a significant decrease in viability to 15%. This result was unexpected because the pH was not reduced in this treatment, causing the fraction of carboxylic acids in the uncharged form to remain low. However, the pH naturally decreased from 6.67 to 5.83 during the exposure period. With the high concentration of spiked acids, there was still a larger concentration of uncharged acids in this treatment compared to Treatments 2, 4, and 5 (**Figure A1.6**), which may have contributed to the decrease in viability. Most surprisingly, Treatment 4 caused the *Ascaris* viability to fall below the detection limit (0.004) (**Figure 3.6**). This result was also replicated in an earlier experiment (**Figure A1.7**). Treatment 4 had no spiked acids or pH adjustment, so no decrease in viability was expected. The only difference between Treatments 4 and 5 was that the HFM in Treatment 5 was autoclaved before adding *Ascaris* eggs to prevent biological activity during the exposure period. We may also assume that biological activity was inhibited in Treatment 2 due to the low pH environment. For Treatment 4, no inoculum was used to promote chain elongation, but an increase in acetic acid, *n*-butyric acid, and *n*-caproic acid provided evidence that some fermentation occurred (**Figure A1.6**). The concentrations of *n*-butyric acid and *n*-caproic acid never reached the levels that we observed in Treatments 1 or 3, and longer chain carboxylic acids, such as *n*-caprylic acid, were not detected (detection limit= 0.2 mM), so we cannot conclude that carboxylic acids caused the observed inactivation. We also do not expect that ammonia contributed to inactivation because the pH of Treatment 4 was 6.4, and ammonia is effective in the NH_3 form, which predominates above pH 9 (Pecson and Nelson, 2005).

Nevertheless, Treatment 4 proved to be the most effective treatment in this experiment. Based on the data collected, we can conclude that an unknown biological process contributed to inactivation and that this process was inhibited by low pH (Treatment 2 versus Treatment 4) and spiking of carboxylic acids (Treatments 1 and 3 versus 4).

Despite the unexpected results, Experiment 4 successfully demonstrated that *n*-butyric acid and *n*-caproic acid are effective at reducing *Ascaris* egg viability within a HFM matrix (Treatment 1 versus Treatments 2 and 5) and that reducing the pH below or near the pK_a of the acids causes greater inactivation (Treatment 1 versus Treatment 3). Because the low pH without carboxylic acids in Treatment 2 inhibited the biological activity that caused inactivation in Treatment 4, we can assume that the low pH in Treatment 1 also inhibited the same biological activity. Therefore, we can conclude that the inactivation observed in Treatment 1 was due to the presence of uncharged carboxylic acids. Although viability was not reduced below the detection limit in this study, we expect that > 99.9% inactivation of *Ascaris* eggs is possible with higher concentrations of uncharged carboxylic acids or a longer exposure time.

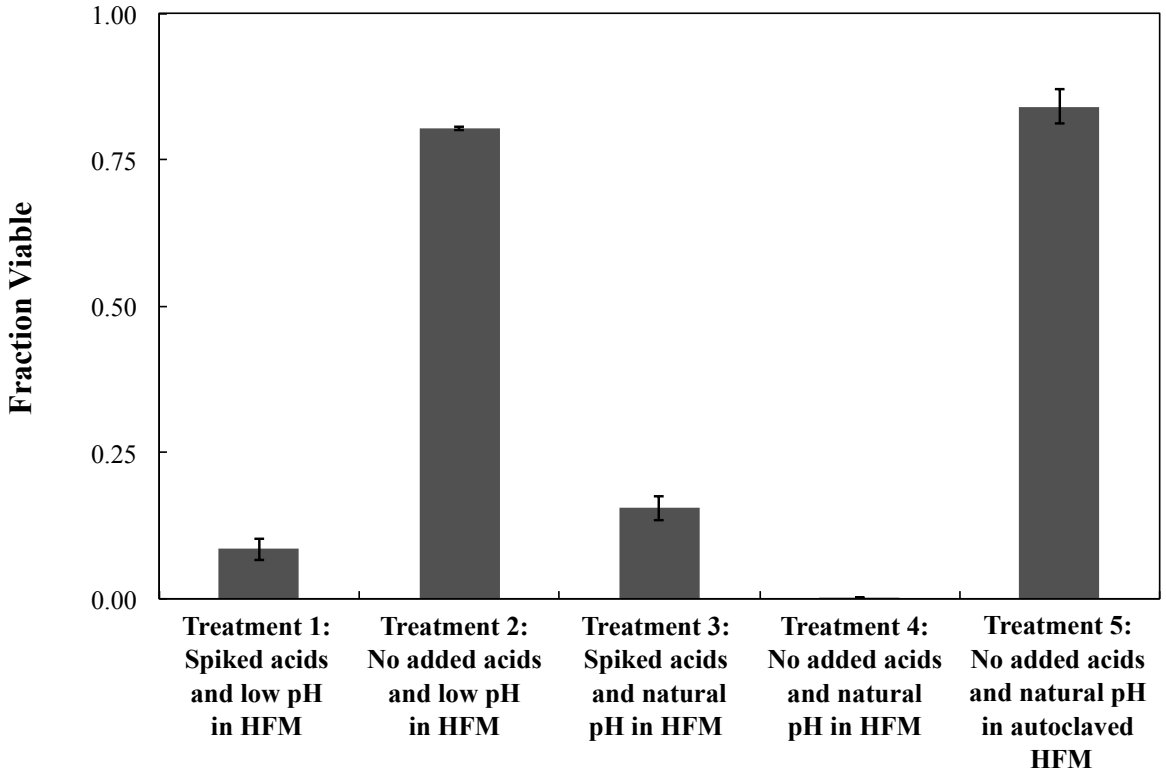

Figure 3.6 Inactivation of *A. suum* eggs in HFM. *A. suum* eggs were exposed to treatment conditions in HFM for 19 days at 30°C. Error bars represent standard deviations of viability for three replicates. Fraction viable for Treatment 4 was below the detection limit of 0.002.

3.4.5 Future work

This study led to greater understanding of the factors that affect carboxylic acid toxicity to *Ascaris* eggs. We have shown that biologically produced concentrations of *n*-butyric acid and *n*-caproic acid are capable of inactivating *Ascaris* eggs. However, the pH must be reduced in fermentation systems to achieve successful inactivation of *Ascaris* eggs at the concentrations of *n*-butyric acid and *n*-caproic acid that were accumulated in Experiment 1. Many studies have shown that anaerobic fermentation of food waste causes a rapid decline in pH (Jiang et al., 2013; Liang and Wan, 2015; Wang et al., 2014), and one study found that co-digesting HFM with food waste caused the pH to drop

below 4 within 12 h (Rajagopal et al., 2014). A two-stage fermentation system may be useful to accumulate high concentrations of carboxylic acids while minimizing product inhibition. In the first stage, fermentation of HFM can accumulate *n*-butyric acid and *n*-caproic acid at a neutral pH, as demonstrated in Experiment 1 (**Figure 3.1**). In the second stage, an additional substrate with high carbohydrate content, such as food waste, could be added to reduce the pH and push the carboxylic acids into the uncharged acid form that inactivates *Ascaris* eggs.

In addition, we have shown that there is no single inhibitory concentration for carboxylic acids. Instead, inactivation is a function of the concentration of uncharged carboxylic acids, the length of the carbon chain, and exposure time. Future studies should investigate the effect of temperature on inactivation rates. Increased temperature is believed to increase the permeability of the lipid membrane of the egg (Wharton, 1979), which would increase the ability of carboxylic acids to enter the egg. Experiment 4 also showed that more work is needed to understand the many factors that affect viability of *Ascaris* eggs in a matrix of HFM. Future studies, therefore, should examine the interactions of multiple carboxylic acids on inactivation as well as any effects of the matrix itself or additional compounds that may exist or be produced during fermentation of HFM.

3.5 Acknowledgements

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3.6 Supporting information

Supporting information is provided in Appendix 1. It contains seven figures and six tables detailing experimental designs, additional results, and specific viabilities of *Ascaris* eggs.

CHAPTER 4

CURRENT TIME-TEMPERATURE RELATIONSHIPS FOR THERMAL INACTIVATION OF *ASCARIS* EGGS AT MESOPHILIC TEMPERATURES ARE TOO CONSERVATIVE AND MAY HAMPER DEVELOPMENT OF SIMPLE, BUT EFFECTIVE SANITATION

Adapted from: Harroff, L.A., Liotta, J.L., Bowman, D.D., Angenent L.T. In review. Current time-temperature relationships for thermal inactivation of *Ascaris* eggs at mesophilic temperatures are too conservative and may hamper development of simple, but effective sanitation. Submitted to *Water Research*.

4.1 Abstract

Ascaris eggs are commonly used as indicators for pathogen inactivation during the treatment of fecal sludge and wastewater due to their highly resistant lipid membrane and ability to survive in the environment for long periods of time. Current guidelines suggest that thermal treatment alone cannot inactivate *Ascaris* eggs at temperatures below 45°C, although some evidence in the literature suggests this to be incorrect. Here, we performed a controlled experiment to test the effect of mesophilic temperatures on *Ascaris* inactivation. We exposed *Ascaris suum* eggs to a temperature gradient between 34°C-45°C under anaerobic and aerobic conditions to observe the required exposure times for a 3-log reduction. Indeed, temperatures lower than 45°C alone did inactivate these eggs. Results from the anaerobic exposures were used to develop a time-temperature relationship that is appropriate for *Ascaris* inactivation at mesophilic temperatures. Data

from the literature demonstrated that our relationship is conservative, with faster inactivation occurring under environmental conditions when *Ascaris* eggs were suspended in fecal sludge or manure. A specific aerobic relationship was not developed, but we demonstrated that aerobic conditions cause faster inactivation than anaerobic conditions. Therefore, the anaerobic relationship provides a conservative guideline for both conditions. We demonstrate clearly that relatively low temperatures can considerably impact *Ascaris* viability and suggest that mesophilic temperatures can be used in waste treatment processes to inactivate pathogens. The development of safe, low-input, mesophilic treatment processes is particularly valuable for ensuring universal access to safe sanitation and excreta management.

4.2 Introduction

The intestinal roundworm *Ascaris lumbricoides* is widely considered to be the most resistant pathogen to disinfection processes in human fecal sludge and wastewater (Feachem et al., 1983; U.S. EPA, 2003). *A. lumbricoides* infects approximately 1.3 billion people worldwide and is spread by fecal-oral transmission, particularly in areas with poor access to sanitation (De Silva et al., 1997). Eggs are passed into the environment through feces and have been shown to survive and retain infectivity for many years (Brudastov et al., 1970). Due to their extreme resistance, *Ascaris* eggs are often used as indicators of pathogen inactivation when testing treatment methods for wastewater and fecal sludge.

The resistance of *Ascaris* eggs to thermal inactivation is of particular interest here. Time-temperature recommendations for using heat to inactivate fecal pathogens are

provided by Feachem et al. (1983) and the U.S. Environmental Protection Agency (US EPA) 40 CFR Part 503 regulations (U.S. EPA, 2003). Both guidelines consider *Ascaris* eggs to be among the most resistant pathogens to thermal inactivation (Feachem et al., 1983; U.S. EPA, 2003). According to the US EPA regulations, *Ascaris* viability is not substantially reduced at temperatures between 32°C and 38°C, and complete reduction at 38°C-50°C is still not guaranteed (U.S. EPA, 2003). Therefore, Feachem et al. and the US EPA suggest a minimum temperature of 45°C and 50°C, respectively, for any thermal treatment, disqualifying many treatment options such as mesophilic (< 45°C) anaerobic or aerobic digestion (Feachem et al., 1983; U.S. EPA, 2003).

Previous work already demonstrated that the Feachem and US EPA guidelines were extremely conservative at thermophilic temperatures (Aitken et al., 2005; Popat et al., 2010). Manser et al. (2015) demonstrated that mesophilic inactivation was feasible with *Ascaris* inactivation observed at 35°C after exposure times of 16-24 days in anaerobic digesters and in phosphate buffer solutions. Several additional studies have investigated the effect of temperature on inactivation by other measures such as ammonia, pH, and peracetic acid (Fidjeland et al., 2015; Ghiglietti et al., 1995; Maya et al., 2012; Nordin et al., 2009; Pecson et al., 2007). All of these studies show that small changes in mesophilic temperatures have a large effect on inactivation rates, and the control treatments from some of these studies can also be used to examine the effect of temperature alone. The combined evidence from these studies clearly indicates that the recommended minimum temperature threshold for *Ascaris* inactivation is too high, but little work has been performed to decisively prove this or to establish a time-temperature relationship that is appropriate for mesophilic temperatures.

The Feachem and US EPA guidelines also make no distinction between time-temperature requirements for anaerobic and aerobic conditions, but inactivation has been found to be faster under aerobic conditions than under anaerobic conditions (Manser et al., 2015). Embryonic development of larvae occurs only in the presence of oxygen, and the eggshell is expected to weaken during this process, leaving the larvae more vulnerable to inactivation. This process is further supported by studies that have found faster inactivation of embryonated eggs compared to unembryonated eggs (Maya et al., 2012). The effect of anaerobic versus aerobic conditions may be particularly important at mesophilic temperatures compared to thermophilic temperatures because longer exposure times will be required during which embryonic development can occur.

Current beliefs about the temperature limits for thermal inactivation may be preventing development of useful, low-input treatment processes that could help provide safe excreta management for the 4.5 billion people that currently live without it (WHO and UNICEF, 2017). To better understand these temperature limits, we exposed *Ascaris suum* eggs to temperatures between 34°C and 45°C for a range of exposure times under anaerobic and aerobic conditions. We used this data to: 1) determine exposure times required to achieve a 3-log reduction of *Ascaris* viability at each temperature; 2) to develop a new time-temperature relationship that can be used to predict *Ascaris* inactivation in this range; and 3) to compare with evidence from the literature to confirm that our relationship is conservative but reasonable. While conditions in the field may require more conservative treatment parameters than those found here, our evidence encourages further study of affordable, low-input mesophilic treatment options for safely managing fecal sludge and wastewater.

4.3 Methods

A. suum eggs were collected from fecal material in the intestines of naturally infected pigs and used as surrogates for *A. lumbricoides* in this study. Collected fecal material was passed through a series of six sieves ranging from US 10-US 100 to remove large debris, and eggs were collected on a US 500 sieve. The eggs were later purified from additional small debris by centrifugal flotation in a magnesium sulfate solution (specific gravity = 1.2) at 800 × g for 5 min. Eggs were stored at 4°C in 0.1 N H_2SO_4 until use.

A series of four exposure trials was conducted, with results from earlier trials used to select appropriate treatment conditions in subsequent trials (**Table 4.1**). Temperatures ranged between 34°C and 45°C. Temperatures below 34°C were not tested because *Ascaris* eggs have been shown to form mobile larvae when incubated aerobically at temperatures up to 34°C (Arene, 1986). For each treatment, 1000 *A. suum* eggs were suspended in 1.5 mL of 0.1 N H_2SO_4 in a 2.0-mL microcentrifuge tube. Anaerobic and aerobic conditions were tested. The tube caps for all treatments were left open and sealed with Parafilm (Bemis NA; Neenah, WI) to allow for gas exchange. Tubes for anaerobic treatments were kept in gas-tight chambers that were made anaerobic using GasPak EZ Anaerobe Sachets (BD, Sparks, MD), and indicator strips (BD, Sparks, MD) were used to confirm anaerobic conditions. Tubes were stored in the dark in incubators, and temperatures were monitored using data loggers (HOBO UX100-003, Bourne, MA and Lascar Electronics EL-USB-1, Eerie, PA). All treatments were performed in duplicate.

After the designated exposure times, eggs were removed from incubators and transferred to 24-well plates, where they were suspended in 0.1 N H_2SO_4 and incubated

aerobically at 28°C for 3 weeks in the dark to promote larval development. Eggs were examined microscopically before and after the 3-week incubation. For eggs treated under anaerobic conditions, pre-incubation examination confirmed that they had not begun developing during treatment, which would have indicated oxygen exposure. For all treatments, viability was quantified after the 3-week incubation period. Approximately 500 eggs were examined in each well. Eggs that had formed larvae were considered viable, and all others were considered nonviable. If no viable eggs were found in the initial 500, the remaining eggs in the well were examined, to a maximum of 1000 eggs. Reported percent viabilities are normalized to the baseline viability of the *A. suum* eggs used for a given trial (**Table 4.1**). Baseline viability was determined by incubating *A. suum* eggs aerobically at 28°C for 3 weeks with no prior treatment.

Table 4.1 Summary of treatment conditions.

Trial	Baseline Percent Viability (SE[1])	Temperature (°C)	Anaerobic/Aerobic	Exposure Times (d)
1	73.6 (2.1)	36	Anaerobic	10, 15, 20, 24, 30, 42
1	73.6 (2.1)	36	Aerobic	10, 15, 20, 24, 30
2	67.5 (1.6)	40	Anaerobic	4, 8, 12, 16
2	67.5 (1.6)	40	Aerobic	2, 4, 8, 12
2	67.5 (1.6)	45	Anaerobic	0.75, 2, 4, 5
2	67.5 (1.6)	45	Aerobic	0.75, 1, 2, 4
3	67.5 (1.6)	36	Aerobic	2, 5, 8, 12
4	69.1 (1.2)	34	Aerobic	3, 5, 10, 15, 20
4	69.1 (1.2)	37	Anaerobic	6, 11, 16, 21, 40
4	69.1 (1.2)	39	Anaerobic	5, 11, 16, 21, 32

[1] Standard error of replicate samples tested for baseline viability. Three samples of 500 eggs were counted for Trial 1, and two samples of 500 eggs were counted for Trials 2-4.

When chemically or thermally treating *Ascaris* eggs, a lag period is expected with minimal loss of viability followed by a region of exponential inactivation (Nordin et al., 2009; Pecson et al., 2007). For most treatments in the present study, the initial sampling

time occurred after the lag period. Therefore, only exponential decline was observed, and inactivation was modeled using linear regression,

$$Log_{10}\left(\frac{N_0}{N}\right) = kt + b \qquad \textbf{(Equation 4.1)}$$

where N_0 is the baseline percent viability of the *A. suum* eggs used for the trial (**Table 4.1**), N is the percent viability of the *A. suum* eggs exposed to a given temperature for time t in days, k is the first-order inactivation constant, and b is the y-intercept, which gives some indication of the expected but unobserved lag period. If viability was below the detection limit for multiple exposure times, then only the shortest time was used in the regression analysis, and viability at that time was assumed to equal the detection limit. The linear regression equations for each temperature were then used to estimate the exposure time required to achieve a 3-log reduction in viability (t_3).

Required inactivation times were also calculated for each temperature according to the equations used in the Feachem guidelines and the US EPA Part 503 guidelines for producing Class A biosolids (Feachem et al., 1983; U.S. EPA, 2003). Feachem et al. (1983) provides a figure with a "zone of safety" of time-temperature combinations that are expected to cause inactivation of *Ascaris* eggs based on literature studies, but the detection limits or degrees of inactivation in these studies are not known. The equation for the boundary of the "zone of safety" is also not given but was derived elsewhere (**Equation 4.2**), where $t_{Feachem}$ is the required exposure time in hours, and T is the temperature in °C (Vinnerås et al., 2003).

$$t_{Feachem} = (1.77 \times 10^2) \times 10^{-0.1944(T-45)} \qquad \textbf{(Equation 4.2)}$$

The US EPA recommended exposure time was calculated based on the time-temperature equation for sewage sludge with at least 7% total solids (**Equation 4.3**), where t_{EPA} is the required exposure time in days, and T is the temperature in °C (U.S. EPA, 2003).

$$t_{EPA} = (1.317 \times 10^{8}) \times 10^{-0.14T} \qquad \textbf{(Equation 4.3)}$$

Equation 4.3 is expected to create Class A biosolids that contain less than 1 viable *Ascaris* egg per 4 g biosolids (dry weight basis). The corresponding log reduction would depend on the initial concentration of viable eggs in the biosolids. It should be noted that the Feachem equation and the US EPA equation were only intended for use at temperatures ≥ 45°C and ≥ 50°C, respectively, but we have extended them here below the recommended minimums for comparison purposes.

Finally, a time-temperature relationship for *Ascaris* inactivation at mesophilic temperatures was developed based on linear regression of the base-ten logarithms of the anaerobic t_3 values versus temperature. The upper limit of the 95% confidence interval for each predicted t_3 value was used to develop a conservative relationship. A literature review was performed to compile additional evidence of *Asscaris* inactivation at temperatures below 45°C. Our relationship was compared to the Feachem relationship, US EPA guidelines, and relevant data points from literature. All statistical analysis was performed using R (R Core Team, 2018), and confidence intervals were calculated using the chemCal package (Ranke, 2018).

4.4 Results and discussion

4.4.1 Ascaris *eggs are inactivated at temperatures and exposure times below current guidelines.*

Anaerobic results

Experimental observations and predicted inactivation times by linear regression (t_3) show that *Ascaris* eggs were inactivated at mesophilic temperatures between 34°C and 45°C when no additional inactivating agents were present (**Figure 4.1**), which directly contradicts guidance from Feachem et al. (1983) and the US EPA (2003). We observed rapid inactivation under anaerobic conditions at 45°, with viability reduced to 26.8% (0.574 log reduction) after only 18 hours and viability equal to or below the detection limit after 2 days (**Figure 4.1A**). In contrast, the US EPA does not expect consistent inactivation of *Ascaris* eggs at 45°C, regardless of the exposure time, and the Feachem relationship predicts a required exposure time of 7.4 days to achieve adequate inactivation (**Table 4.2**) (Feachem et al., 1983; U.S. EPA, 2003). At 40°C under anaerobic conditions, we observed an average maximum log reduction of 2.19 after a 16-day exposure time (**Figure 4.1A**), and the predicted t_3 from linear regression was 20.5 days (**Table 4.2**). Although the t_3 value is extrapolated past the observed data in this case, we have high confidence in the regression because all four time points occurred along the exponential portion of the inactivation curve (**Figure 4.1A**), and the regression provided a good fit to the data with all regression parameters significant at $p < 0.05$ and $R^2 = 0.954$ (**Table 4.2**). Under anaerobic conditions at 39°C, the exponential portion of the inactivation curve was fully captured, and we found no viable eggs after a 32-day exposure time (**Figure 4.1A**). Our linear regression fit the data well and also predicted 32

days as the required exposure time for a 3-log reduction (**Table 4.2**). Meanwhile, the extended Feachem and US EPA relationships predict much longer required exposure times of 108 and 457 days, respectively (Feachem et al., 1983; U.S. EPA, 2003).

The longest exposure time tested under anaerobic conditions at 37°C was 40 days, at which point the results from the duplicate samples varied. Viability was reduced below detection (2.84 log reduction) for one sample, but only a 1.37 log reduction was observed for the second sample (**Figure 4.1A**). Correspondingly, the t_3 predicted by linear regression (57.5 days) was longer than 40 days (**Table 4.2**). The regression line fit the data well (R^2= 0.759) but would be improved with an additional time point beyond 40 days to demonstrate viability below detection in both replicates and an additional point between 21 and 40 days to refine the rate of decline. Unfortunately, the three-week incubation time required between the end of the exposure period and examining the eggs for viability prevented us from recognizing the need for these treatments until after the experiment was completed. Longer exposure times were also needed to improve inactivation predictions under anaerobic conditions at 36°C. The longest exposure time we tested was 42 days, and we observed minimal log reductions of only 0.298 and 0.323, indicating that the exponential portion of the inactivation curve was not observed (**Figure 4.1A**). As expected, the resulting linear regression provided a poor fit to the data (**Table 4.2**), and we did not use the t_3 when developing our time-temperature relationship.

Aerobic results

For all temperatures tested under aerobic conditions, *Ascaris* viability was reduced below detection during the tested exposure times. Inactivation at 45°C under aerobic conditions

was similar to the inactivation observed under anaerobic conditions with no viable eggs found after a 2-day exposure time (**Figure 4.1B**). For temperatures between 34°C and 40°C, mesophilic inactivation of *Ascaris* eggs was even more effective under aerobic conditions than under anaerobic conditions (**Figure 4.1B**). Under aerobic conditions at 40°C no viable eggs were found after an exposure time of 8 days, although low viability was still observed after 12 days (average = 0.5%), and exposure times longer than 12 days were not tested (**Figure 4.1B**). The t_3 predicted for 40°C was 12.7 days (**Table 4.2**), which was significantly faster than 69.2 days and 331 days, as estimated by the extended Feachem and US EPA relationships, respectively (Feachem et al., 1983; U.S. EPA, 2003). Inactivation rates under aerobic conditions at 34°C and 36°C were similar, with no viable eggs found after exposure times of 15 days for both temperatures (**Figure 4.1B**). This result was 25-150 times faster than the inactivation times estimated by the extended Feachem and US EPA relationships (**Table 4.2**).

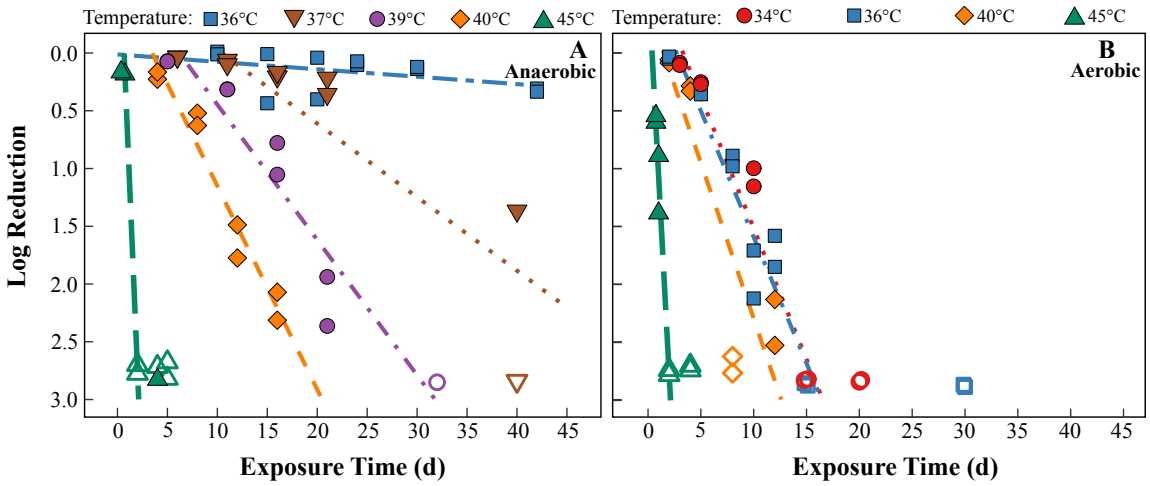

Figure 4.1 Log reduction of *Ascaris* viability when exposed to mesophilic temperatures between 34°C and 45°C under anaerobic (A) and aerobic (B) conditions. Duplicate samples for each treatment are shown individually. Open symbols indicate that no viable eggs were found and are plotted as equal to the detection limit (average detection limit = 2.81). Lines represent inactivation curves estimated for each temperature using linear regression. Raw data for each treatment is included as supplemental information (**Table A2.1**).

Table 4.2 Results from linear regression of experimental data, inactivation times predicted by the linear regression (t_3), and inactivation times predicted by three time-temperature relationships ($t_{Feachem}$, t_{EPA}, and $t_{Harroff}$). Standard errors for linear regression parameters are given in parentheses. Exposure time required for a 3-log reduction (t_3) was calculated using the regression parameters, and the lower and upper bounds of the 95% confidence interval are given in parentheses. The t_3 values from anaerobic observations at 37°C-45°C were used to develop our time-temperature relationship, and the corresponding predicted times required for a 3-log reduction are given as $t_{Harroff}$. Inactivation times required by the Feachem ($t_{Feachem}$) and US EPA (t_{EPA}) guidelines are also given as comparison.

Anaerobic/ Aerobic	Temperature (°C)	Linear regression of experimental data				Inactivation times predicted by time-temperature relationships		
		k (SE)	*b* (SE)	R^2	t_3 (d) (95% CI)	$t_{Feachem}$ [c] (d)	t_{EPA} [d] (d)	$t_{Harroff}$ [e] (d)
Anaerobic	36	0.00642 [a] (0.00424)	0.0124 [a] (0.109)	0.187	465 [b] (-187; 1120)	414	1201	151
	37	0.0637 (0.0127)	-0.663 (0.282)	0.759	57.5 [b] (32.4; 82.7)	265	870	95.4
	39	0.117 (0.0142)	-0.724 (0.246)	0.907	31.8 (23.2; 40.4)	108	457	38.1
	40	0.176 (0.0158)	-0.612 (0.172)	0.954	20.5 [b] (16.8; 24.2)	69.2	331	24.1
	45	2.05 (0.0305)	-1.35 (0.0461)	0.999	2.12 (2.03; 2.23)	7.38	66.0	2.44
Aerobic	34	0.226 (0.0253)	-0.746 (0.235)	0.930	16.6 (12.1; 21.1)	1014	2289	377
	36	0.218 (0.0189)	-0.586 (0.183)	0.930	16.5 (13.1; 19.8)	414	1201	151
	40	0.268 (0.0610)	-0.391 [a] (0.459)	0.763	12.7 (5.40; 19.9)	69.2	331	24.1
	45	1.71 (0.144)	-0.650 (0.196)	0.973	2.12 (1.74; 2.52)	7.38	66.0	2.44

[a] Parameter is not significant at $p < 0.05$.
[b] Viability was not reduced below the detection limit during the exposure times tested. Inactivation times are therefore extrapolated.
[c] (Feachem et al., 1983)
[d] (U.S. EPA, 2003)
[e] Current study

4.4.2 We developed a time-temperature relationship for mesophilic temperatures and anaerobic conditions that is reasonable and conservative.

Our results demonstrate that thermal inactivation of *Ascaris* eggs consistently occurs at temperatures below 45°C and that the current guidelines significantly over-predict the

times required to achieve inactivation at these temperatures (**Table 4.2**). Therefore, a new time-temperature relationship specific for temperatures between 34°C and 45°C is clearly necessary. We developed this relationship using linear regression of the base-ten logarithm of our anaerobic t_3 values as function of temperature, rearranged to produce **Equation 4.4**,

$$t_{Harroff} = (2.20 \times 10^9) \times 10^{-0.199T} \qquad \textbf{(Equation 4.4)}$$

where $t_{Harroff}$ is the exposure time required for a 3-log reduction in days, and T is temperature in °C. The upper limit of the 95% confidence interval was used for each predicted t_3 value to develop a more conservative relationship, and the t_3 estimated for anaerobic inactivation at 36°C was excluded due to the poor fit of the linear regression model.

We compared our time-temperature relationship to the Feachem and US EPA guidelines and to mesophilic inactivation that has been observed in literature under anaerobic conditions (**Table 4.2, Figure 4.2A**) (Cruz Espinoza et al., 2012; Johansen et al., 2013; Manser et al., 2015; Nordin et al., 2009; Pecson et al., 2007; Scheinemann et al., 2015). We used literature data points that measured *Ascaris* viability without addition of external inactivating agents (e.g., ammonia or volatile fatty acids). Therefore, inactivation is assumed to occur primarily due to temperature, although some variation is expected due to differences in moisture content, sludge properties, and intrinsic concentrations of inactivating compounds (Cruz Espinoza et al., 2012; Senecal et al., 2018). One additional study observed inactivation at 42°C after 6 days with eggs suspended in a mixture of phosphate buffer and fecal material, but it was not included in **Figure 4.2A** because the presence of anaerobic or aerobic conditions was not clear

(Senecal et al., 2018). Many of the relevant literature data points were control treatments from larger studies. Individually, these data points prove little about mesophilic thermal inactivation, but they provide convincing evidence when combined. Details about each literature data point in **Figure 4.2**, as well as other studies that are discussed here, are included as supplementary data (**Table A2.2**). Details in **Table A2.2** include exposure conditions, how inactivation times were determined, and the degree of inactivation that was measured in each study.

With all literature data points falling at or below our time-temperature relationship and our experimental data points, we conclude that the time-temperature relationship developed here is more reasonable for temperatures below 45°C than the Feachem and US EPA relationships, while still being conservative. The conservative nature of our time-temperature relationship can be attributed to the use of aqueous solutions for exposing eggs in our experimental results versus the use of manure or fecal sludge matrices for the literature points. Past work looking at *Ascaris* inactivation in autoclaved versus non-autoclaved anaerobic fecal material at 30°C showed that compounds may be biologically produced during anaerobic fermentation that contribute to *Ascaris* inactivation (Harroff et al., 2017). Other studies examining *Ascaris* inactivation by ammonia have also found that results from exposures in aqueous solutions are conservative compared to those in manures and fecal sludge (Nordin et al., 2009; Pecson et al., 2007; Schuh et al., 1985). Here, the relationship that we developed using aqueous exposures appears more conservative at lower temperatures than at higher temperatures, indicating that intrinsic inactivating compounds in sludge became less

important at warmer temperatures within the mesophilic spectrum (Fidjeland et al., 2015).

Even though overly conservative guidelines may hamper the use of simple treatment, the use of a conservative time-temperature relationship is still important for protecting public health when developing waste treatment strategies. For this reason, our time-temperature relationship should still be confirmed at temperatures less than 37°C using exposures in aqueous solutions. Although literature values demonstrate inactivation at 34°C and 35°C at exposure times less than those predicted by our relationship (Cruz Espinoza et al., 2012; Manser et al., 2015; Nordin et al., 2009), intrinsic factors in those treatments, such as ammonia and solids concentration, may have affected inactivation times. For example, Manser et al. (2015) observed 99% inactivation of *Ascaris* eggs after 24 days in an anaerobic digester at 35°C, but another study observed only 50% inactivation after 35 days in a 35°C digester (Johnson et al., 1998). Both times are far below the 238 days required for a 3-log (99.9%) inactivation by our time-temperature relationship, but they suggest a need for more information.

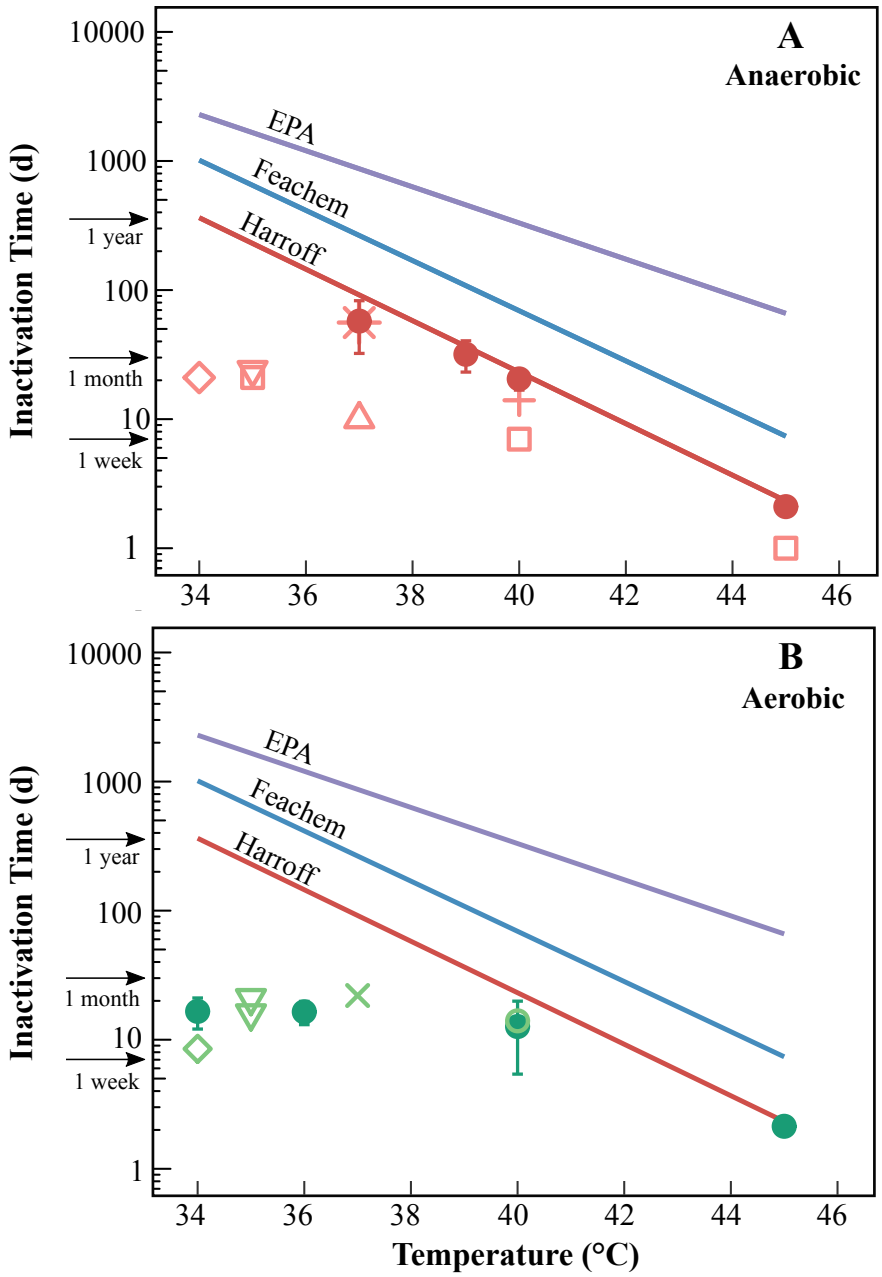

Figure 4.2 Proposed time-temperature relationship for a 3-log reduction of *Ascaris* eggs at 34°C-45°C. Our relationship (Harroff) is compared to the Feachem and US EPA guidelines. Data points show t_3 values from this study (●) with error bars representing the 95% confidence interval. Literature data points are also shown from (Cruz Espinoza et al., 2012) (□), (Ghiglietti et al., 1995) (○), (Johansen et al., 2013) (△), (Manser et al., 2015) (▽), (Nordin et al., 2009) (◇), (Pecson et al., 2007) (+), (Scheinemann et al., 2015) (✳), and (Tharaldsen and Helle, 1989) (×). All data points in panel A (red) were observed under anaerobic conditions, and all data points in panel B (green) were observed under aerobic conditions.

4.4.3 Aerobic conditions decrease required inactivation times, particularly at lower temperatures.

Our time-temperature relationship, which we obtained at strict anaerobic conditions, is conservative for another reason that becomes apparent in this section. Oxygen improves the inactivation rates, so any oxygen intrusion will reduce the inactivation times. Recent work by Manser et al. (2015) compared inactivation times required for *Ascaris* inactivation under anaerobic and aerobic conditions at 34°C (24 days and 16 days, respectively) and concluded that aerobic conditions cause faster inactivation. An earlier study compared anaerobic and aerobic digestion at 37°C and 47°C and reached the opposite conclusion that anaerobic conditions caused faster inactivation (Kato et al., 2003). However, the authors acknowledged that the actual temperatures in the aerobic digesters were uncertain due to the use of circulating air to keep them oxygenated, leading to inconclusive results (Kato et al., 2003). Our experimental data supports the conclusion of Manser et al. (2015) at a range of mesophilic temperatures between 34°C and 40°C (**Figure 4.1**), which has not been shown before. In addition, our data shows that the effect of oxygen exposure decreases as temperature increases. Three temperatures (36°C, 40°C, and 45°C) were tested under both anaerobic and aerobic conditions. We cannot directly compare the 36°C treatments because exponential decline was not observed under anaerobic conditions, but we observed that the t_3 for 37°C under anaerobic conditions is 3.5 times larger than the t_3 predicted for 36°C under aerobic conditions (**Table 4.2**). Meanwhile, at 40°C the t_3 under anaerobic conditions is only 1.6 times larger than the t_3 under aerobic conditions (**Table 4.2**). The t_3 values at 45°C for anaerobic and aerobic conditions are equal, which indicates that the effect of oxygen

becomes negligible at this temperature (**Table 4.2**). Aerobic conditions cause faster inactivation because the larvae undergo embryonic development when they have access to oxygen, and they consume lipids and carbohydrates within the eggshell to cause structural and chemical changes that may leave the larvae more susceptible to environmental conditions (Arene, 1986; Fairbairn, 1957; Manser et al., 2015). However, inactivation at 45°C is likely too rapid (2.1 days) to allow for significant embryonic development, which causes the effect of oxygen presence to be negated.

Previous studies have shown aerobic inactivation at times and temperatures consistent with ours, but they did not directly compare inactivation rates under anaerobic and aerobic conditions (**Figure 4.2B, Table A2.2**). In control treatments of a pH 7 saline solution, Ghiglietti et al. (1995) observed 99% inactivation of *Ascaris* eggs after exposure to 40°C for 14 days (**Figure 4.2B**). Aerobic conditions were not explicitly indicated in the Ghiglietti study, but embryonic development was observed during the exposure time for treatments at lower temperatures so we can assume that oxygen was present in the treatments (Ghiglietti et al., 1995). In comparison, our t_3 for 40°C under aerobic conditions was slightly lower at 12.7 days. Tharaldsen and Helle (1989) found that *Ascaris* eggs in mechanically aerated pig manure slurry at 37°C were 99% inactivated between 14 and 22 days, which is shown in **Figure 4.2B** as 22 days, and we observed similar inactivation at 36°C with a predicted t_3 of 16.5 days.

Nordin et al. (2009) examined *Ascaris* inactivation from different ammonia concentrations at 34°C and reported results that also indicate an effect of aerobic conditions. In one treatment of fecal material containing 43 mM aqueous ammonia, 99% inactivation was predicted after 21 days using linear regression. For a treatment using

diluted urine containing 40 mM aqueous NH_3, 99% inactivation was predicted after only 8.5 days. With similar ammonia concentrations, the study concluded that unmonitored factors contributed to the different inactivation rates. We propose here that oxygen was the primary unmonitored factor. For the fecal material treatment, eggs were contained in mesh bags and inserted in 200 g of fecal material in sealed containers, leading to anaerobic conditions. For the urine treatment, the mesh bags were suspended in urine contained in a sealed 50-mL tube. However, the tube was opened on days 1, 2, 7, and 10 for sample collection, allowing the urine to be oxygenated each time and decreasing the required exposure time for inactivation. A control treatment of 0.9% NaCl solution at the same temperature also provided an interesting result because eggs were only 7% inactivated after 31 days. Based on our experimental results, we expect eggs in aerobic conditions at 34°C to be 99.9% inactivated after 16.6 days. However, results for the control are only reported after 31 days. Without the frequent sampling interval used for the urine treatment, the control would have been anaerobic, and we would not expect to see significant inactivation after 31 days. Results reported for the urine treatment are expectedly faster than ours due to the added inactivating effects of ammonia in the urine.

Other studies have examined the effect of temperature on rates of embryonic development in *Ascaris* and found that eggs could not fully develop into motile larvae at temperatures between 34°C and 45°C (Arene, 1986; Gaspard et al., 1996; Seamster, 1950). We cannot predict the required exposure times for these studies because the eggs were never removed from the warmer temperatures, but embryonic development is expected to occur within 14-21 days, and therefore we can assume that required exposure times would be less than 21 days for temperatures between 34°C and 45°C. Similarly, all

of our predicted inactivation times for the same temperature range under aerobic conditions were less than 21 days.

Ideally, a separate time-temperature relationship would be developed for *Ascaris* in the presence of oxygen. However, more experimental data is needed to accurately capture the effect of oxygen and how it increases at lower temperatures. When we compare our data points under aerobic conditions, literature data points under aerobic conditions, and our data points under anaerobic conditions, we confirm that anaerobic conditions present a conservative case (**Figure 4.2B**). This has important implications when designing sanitation and waste treatment processes because aerobic processes would require shorter treatment times than the time-temperature relationship would predict. These results also demonstrate the importance of strictly maintaining and monitoring anaerobic and aerobic conditions when testing *Ascaris* inactivation by temperature and other mechanisms. Inactivation that is observed under aerobic conditions may not occur under anaerobic conditions. Monitoring of additional pathogens may also be required for mesophilic treatment systems, particularly under aerobic conditions. Previous work has shown that *Escherichia coli* and *Salmonella* are also inactivated faster under aerobic conditions than under anaerobic conditions (Pandey et al., 2016), but the extent to which different pathogens are affected is not known, and it is possible that other pathogens will prove more resilient than *Ascaris* eggs under certain conditions.

4.4.4 Limitations

Caution must be used when applying the results and time-temperature relationship shown here to large-scale systems, where required exposure times for inactivation may be

affected by uneven heating, fluctuating temperatures, and shielding effects of solids (Popat et al., 2010; Senecal et al., 2018). For example, composting systems often do not reach expected temperatures, leading to poor pathogen inactivation (Mehl et al., 2011). Those that do reach proper temperatures have large spatial and temporal variations that make thermal inactivation difficult to predict. In one study, measured temperatures ranged between < 30°C and nearly 50°C in different portions of a compost heap over a 120 day period, and over 100 days of exposure was required to observe > 99% *Ascaris* inactivation (Jensen et al., 2009). Another study observed temperatures > 60°C. *Ascaris* inactivation would typically be expected within minutes at such high temperatures, but 6 days were required to reduce viability below detection, indicating pockets with a lower temperature (Szabová et al., 2010). In addition to variation within treatment systems, Pecson and Nelson (2005) also have observed variability in temperature resiliency between batches of *Ascaris* eggs. Temperature must be closely controlled and monitored for any treatment system relying on thermal inactivation of pathogens. For mesophilic systems in particular, pathogen inactivation should be verified for each unique system.

4.5 Conclusions

- Given adequate exposure times, *Ascaris* eggs are consistently inactivated under both anaerobic and aerobic conditions at mesophilic temperatures < 45°C without the addition of external inactivating agents.
- The relationship $t_{Harroff} = (2.20 \times 10^{9}) \times 10^{-0.199T}$ can be used to conservatively estimate exposure times (t, days) required to achieve a 3-log

reduction of *Ascaris* viability at temperatures (T) < 45°C under both anaerobic and aerobic conditions.

- This relationship is still conservative, but much less conservative than current relationships that are used as standards. Therefore, reduction of minimum temperature thresholds in regulations for thermal waste treatment processes should be considered.
- Mesophilic waste treatment processes should be given greater consideration when considering how to treat the excreta of the 4.5 billion people globally who currently lack access to safely managed sanitation services (WHO and UNICEF, 2017). Mesophilic systems can be less expensive and easier to operate than thermophilic systems, and they are shown here to potentially provide adequate pathogen treatment.
- Thermal inactivation of *Ascaris* eggs is faster under aerobic conditions than under anaerobic conditions. Therefore, presence of oxygen should be monitored more closely in research. New waste treatment systems should be developed that use this advantage of oxygen to inactivate pathogens.
- *Ascaris* is less resistant to thermal inactivation than is often stated, and more work is needed to evaluate the circumstances under which it can be used as a reliable indicator organism.

4.6 Acknowledgements

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4.7 Supporting information

Supporting information is provided in Appendix 2. It contains two tables detailing 1) experimental treatment conditions and results and 2) a summary of literature that was reviewed.

CHAPTER 5

DEVELOPMENT OF A MODEL TO PREDICT *ASCARIS* INACTIVATION BASED ON CARBOXYLIC ACID CONCENTRATIONS, EXPOSURE TIME, AND TEMPERATURE

Adapted from: Harroff, L.A., Johnson, L.M., Liotta, J.L., Bowman, D.D., and Angenent, L.A. In review. Development of a model to predict *Ascaris* inactivation based on carboxylic acid concentrations, exposure time, and temperature. Submitted to *Environmental Technology*.

5.1 Abstract

Ascaris eggs are commonly used as indicators for pathogen inactivation in fecal sludge and wastewater treatment methods due to their tremendous resistance to disinfection processes. Previous work has shown that *Ascaris* eggs, and the pathogens they indicate, are inactivated by carboxylic acids, which can be produced *in situ* through anaerobic fermentation of human fecal material (HFM). Here, we developed a model that predicts *Ascaris* viability as function of: (1) concentrations of *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid; (2) exposure time; and (3) temperature. We utilized response surface methodology (RSM) to construct a preliminary linear regression model, but we found that a logistic regression provided a better fit to a larger dataset. The model performs well for predicting viability at a wide range of each factor when *Ascaris* eggs are exposed to aqueous solutions of carboxylic acids, but limited data suggests that the model might over-predict inactivation when eggs are exposed in actual HFM.

5.2. Introduction

With 4.5 billion people lacking access to safely managed sanitation services, innovative methods for treating waste and inactivating pathogens are needed (WHO and UNICEF, 2017). Particular attention should be given to low-input methods that can aid in resource recovery from sanitation waste. One such method utilizes anaerobic fermentation of the solid waste fraction to accumulate carboxylic acids (Harroff et al., 2017). For this bioprocess, open cultures of anaerobic bacteria break down organic wastes into short-chain carboxylic acids (e.g., acetic acid, propionic acid, and *n*-butyric acid) and then elongate the hydrocarbon chains to produce *n*-butyric acid (C4), *n*-caproic acid (C6), and even *n*-caprylic acid (C8) (Agler et al., 2011; Spirito et al., 2014). Acetic acid (C2) is usually the dominant short-chain carboxylic acid after primary fermentation, and chain elongation proceeds with the addition of two carbon molecules at a time. Therefore, carboxylic acids with even-numbered carbon chains are expected to occur in higher concentrations than those with odd-numbered carbon chains (e.g., *n*-valeric acid), although both can occur (Agler et al., 2011). Previous work has shown that human fecal material (HFM) can be used as the sole substrate for chain elongation without requiring additional inputs. During 1-L batch fermentation experiments, concentrations of 257 mM *n*-butyric acid, 11.3 mM *n*-valeric acid, and 27.1 mM *n*-caproic acid accumulated after 46 days (Harroff et al., 2017).

Carboxylic acid production in HFM is particularly interesting because it can be used to inactivate pathogens as part of a waste treatment system (Abdul and Lloyd, 1985; Royce et al., 2013; Skřivanová et al., 2006). Our work uses eggs from the *Ascaris suum*

roundworm as indicators for pathogen inactivation. *A. suum* is a roundworm that infects pigs and can be used as a surrogate for the human roundworm *Ascaris lumbricoides* (Feachem et al., 1983). *A. lumbricoides* is considered the most robust pathogen in human excreta due to protection by the innermost lipid layer of the egg, which is impermeable to most disinfectants (Arfaa, 1978; Feachem et al., 1983). Previous work demonstrated that carboxylic acids can permeate the lipid layer of *Ascaris* eggs to cause inactivation when they are in the undissociated form (Barrios et al., 2007; Butkus et al., 2011; Harroff et al., 2017; Riungu et al., 2018; Rojas-Oropeza et al., 2016). A pH below the pKa of the carboxylic acid (~4.8), therefore, aids in inactivation by shifting the carboxylic acids to the undissociated form. Indeed, previous work has demonstrated that a low pH value affects *Ascaris* inactivation through this indirect effect because the low pH value does not directly cause *Ascaris* inactivation (Harroff et al., 2017). Carboxylic acids with longer chains are also more effective than those with shorter chains due to the hydrophobic nature of the hydrocarbon tail (Butkus et al., 2011; Harroff et al., 2017).

Time and concentration combinations that result in > 99.9% inactivation of *Ascaris* eggs were previously determined for *n*-butyric acid and *n*-caproic acid individually at 30°C (Harroff et al., 2017), but more information is needed to predict inactivation rates in waste treatment processes where: (1) mixtures of carboxylic acids will be present; and (2) temperature will vary. Previous work has shown that temperature has a significant effect on *Ascaris* inactivation rates by ammonia, with a temperature increase of 16.1°C causing a 10-fold decrease in the required exposure time (Fidjeland et al., 2015). Temperature is expected to increase the permeability of the lipid layer of the eggs to allow NH_3 to pass more easily (Fidjeland et al., 2015; Wharton, 1979). Since

ammonia and carboxylic acid inactivation both rely on the hydrophobicity of the molecules to cross the lipid layer, we expect temperature to affect both processes similarly.

Here, we used response surface methodology (RSM) to evaluate the combined effects of five different factors on *Ascaris* inactivation: (1) concentration of *n*-butyric acid; (2) concentration of *n*-valeric acid; (3) concentration of *n*-caproic acid; (4) exposure time; and (5) temperature. RSM has been used in other studies to examine pathogen inactivation and wastewater treatment processes, and it is useful for exploring the effects and interactions of multiple factors on a single response variable, while maintaining a reasonable number of samples (Bover-Cid et al., 2012; Ghasempur et al., 2007). However, we found that a logistic regression model provided a better fit to our data than the linear regression model typically used with RSM. Further improvements are still required, though, because limited evidence suggests that our model over-predicts inactivation rates when *Ascaris* eggs are exposed in HFM matrices instead of aqueous solutions.

5.3 Methods

5.3.1 Experimental design

Four groups of treatments (Groups 1-4) were tested to evaluate the effect of five factors on *Ascaris* egg viability: (1) *n*-butyric acid concentration (Bu); (2) *n*-valeric acid concentration (Va); (3) *n*-caproic acid concentration (Ca); (4) exposure time (Ti); and (5) temperature (Te) (**Table 5.1**). We use "treatment" to indicate a specific combination of factors, and "sample" to indicate the specific tube in which the *Ascaris* eggs were tested.

The three carboxylic acids were selected based on abundance in fermentation systems and chain length. *n*-Butyric acid and *n*-caproic acid have an even number of carbons and are, therefore, more abundant in chain elongation fermentation systems. *n*-Valeric acid was also included because it was observed in batch fermentations of HFM. The concentration was relatively low, but the longer chain length makes it a more promising candidate for inactivation (Harroff et al., 2017). Acetic acid and propionic acid were also found in fermentation studies, but these acids are ineffective at inactivating *Ascaris* eggs, even at very high concentrations, due to their shorter hydrocarbon chains (Butkus et al., 2011).

Table 5.1 Description of treatments performed in each of four groups.

Group #	Group description	Variable ranges tested				
		n-Butyric acid (mM)	*n*-Valeric acid (mM)	*n*-Caproic acid (mM)	Exposure time (d)	Temp (°C)
1	CCD 1; 8 additional treatments performed in duplicate with no carboxylic acids; 1 additional treatment performed in duplicate with –α temperature and a longer exposure time than required by the CCD	0-500	0-90	0-24	1-30	15-45
2	CCD 2; center point from first CCD repeated six times to evaluate consistency between experiments; 9 additional treatments performed in duplicate with no carboxylic acids	0-260	0-50	0-14	1-19	15-45
3	37 new treatments tested in duplicate (2 samples) using the same concentrations of carboxylic acids from Group 2 with different combinations of temperature and exposure time, all within the ranges tested in Group 2; 3 treatments replicated from Group 2	75-185	14-36	4-10	3-10	24-36
4	13 treatments tested in duplicate with higher carboxylic concentrations more representative of concentrations expected to see in fermentation systems; temperatures and exposure times reduced from Group 1; 2 treatments replicated from Group 3	185-260	12-36	10-27	1-12	23-37

Central composite design (CCD) is an experimental design used for RSM that we utilized in Groups 1 and 2 to test the five factors while maintaining a reasonable number of samples. For a five-factor CCD, only 48 samples are needed, while a full factorial design would require 1024 samples plus replicates. CCDs test each factor at five levels,

which are coded as –α, -1, 0, +1, and +α. Level 0 is the median value between -1 and +1. The interval between the actual values that are represented by +/-1 and 0 can be varied for each factor depending on the range that should be tested. The interval between 0 and +/- 1 is multiplied by 2.38 to get the interval between 0 and +/-α. The multiplication factor of 2.38 is prescribed by the CCD to achieve a rotatable design (Montgomery, 2013). For the CCD in Group 1, levels for the carboxylic acid concentrations were chosen with Level 0 similar to the concentrations found during previous batch fermentation experiments with human fecal material (Harroff et al., 2017), and the intervals between levels were chosen so that the value for -α was 0 mM for each acid (**Table 5.2**). A second CCD was later performed with Group 2 with lower carboxylic acid concentrations and the same exposure times and temperatures (**Table 5.2**). The CCD designates specific combinations of each level to be used as treatments. There were 32 factorial point treatments with each factor at its -1 or +1 value, and all combinations of the +/- 1 values were tested. There was also one center point treatment that was replicated six times. For this treatment, every factor was at Level 0. Finally, there were 10 axial point treatments, which had one factor at its +/- α value and all other factors at 0.

Groups 1 and 2 also contained additional treatments, beyond those required by the CCDs, to test the effects of exposure time and temperature with no carboxylic acids present. Groups 3 and 4 were later added to improve and validate the model (**Table 5.1**). For the 48 CCD samples in Groups 1 and 2, replicates were only performed of the center point treatment, which is standard for CCDs (Montgomery, 2013; Rosenberger, 2018). For the additional treatments tested in Groups 1 and 2 and all treatments in Groups 3 and

4, duplicate samples were used for each treatment, because CCD was not used, for reasons discussed within the results section.

Table 5.2 Variables and levels used for central composite designs (CCDs).

Level	Variables				
	Bu *n*-Butyrate (mM)	Va *n*-Valerate (mM)	Ca *n*-Caproate (mM)	Ti Exposure time (d)	Te Temperature (°C)
CCD 1					
-α	0.0	0.0	0.0	1.0	15.0
-1	145.0	26.1	7.0	6.2	23.7
0	250.0	45.0	12.0	10.0	30.0
1	355.0	63.9	17.0	13.8	36.3
α	500.0	90.0	24.0	19.0	45.0
CCD 2					
-α	0.0	0.0	0.0	1.0	15.0
-1	75.4	14.5	4.1	6.2	23.7
0	130.0	25.0	7.0	10.0	30.0
1	185.0	35.5	9.9	13.8	36.3
α	260.0	50.0	14.0	19.0	45.0

5.3.2 Experimental setup

Ascaris suum eggs were collected from intestinal contents of naturally infected slaughterhouse pigs. These eggs were used as surrogates for *Ascaris lumbricoides*. *A. suum* eggs were separated from fecal material by sieving and centrifugal flotation, as described elsewhere (Harroff et al., 2017), and were stored in 0.1 N H_2SO_4 until use. The baseline viability for the eggs was 72.5%-84.7%.

For each sample, approximately 1000 *A. suum* eggs were suspended in a microcentrifuge tube containing 1.5 mL of a solution with varied concentrations of *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid in DI water. Previous work demonstrated that only undissociated carboxylic acids were effective at inactivating *Ascaris* eggs, and therefore a pH of 2 was used for all solutions to ensure that > 99.8% of the total

concentration was in the undissociated form (Harroff et al., 2017). HCl at a 5-M concentration was used to adjust the pH. All solutions also contained 5 mM acetic acid to act as a pH buffer for treatments that did not contain any other carboxylic acids. Previous work has shown that low concentrations of acetic acid are ineffective at reducing *Ascaris* viability (Butkus et al., 2011).

Sample tubes were kept in sealed containers, which were made anaerobic using GasPak EZ Anaerobe Sachets (BD, Sparks, MD). The tube caps were left open and sealed with Parafilm (Bemis NA; Neenah, WI) to prevent evaporation while allowing gas exchange with the GasPaks. Sealed containers were kept in incubators for exposure temperatures > 20°C. Samples at 15°C were kept in a temperature-controlled refrigerator. After the exposure time, samples were centrifuged at 3500 × g for 4.5 min. The supernatant was collected to measure carboxylic acid concentrations, and the eggs were washed with a sodium phosphate buffer solution, as described in detail previously (Harroff et al., 2017). Eggs were examined microscopically to confirm that larval development was not initiated during the exposure period, indicating that anaerobic conditions had been maintained. The eggs were then incubated aerobically for 3 weeks at 28°C in the dark before examining them microscopically again. At least 500 eggs were counted for each sample, and eggs containing fully formed larvae were considered viable. If no viable eggs were counted in the initial 500 eggs, then the remaining eggs were counted, to a maximum of 1000. The percent viability is reported as the number of viable eggs divided by the total number of eggs counted multiplied by 100.

Temperature was recorded every 10 to 15 min throughout the exposure period using data loggers (HOBO UX100-003, Bourne, MA and Lascar Electronics EL-USB-1,

Eerie, PA), and average temperatures were calculated individually for each sample. Carboxylic acid concentrations were verified by gas chromatography (GC) (HP Agilent 6890, Santa Clara, CA) at the beginning and end of the exposure period for each sample. End point concentrations were measured to capture any changes that might occur due to volatilization of carboxylic acids or evaporation of water. GC analysis was performed with a Nukol fused silica capillary column (15 m × 0.53 mm × 0.50 μm; Supelco Inc., Bellefonte, PA) and flame ionization detector (FID) with helium used as the carrier gas. The method was based on previous work (Usack and Angenent, 2015) with inlet and outlet temperatures at 200°C and 275°C, respectively. The initial oven temperature was 70°C with a ramp of 12°C min^{-1} to a maximum of 170°C, which was held for 2 min.

5.3.3 Model fitting and statistical analysis

All statistical analysis and model development were performed using R (R Core Team, 2018). Measured values of factors (*n*-butyric acid concentration, *n*-valeric acid concentration, *n*-caproic acid concentration, exposure time, and temperature) were used for each sample. For carboxylic acid concentrations, averages of the measured concentrations at the beginning and end of each exposure period were used. For temperature, averages of the temperature measurements recorded throughout the exposure period were used. Exposure times were calculated based on the time that samples were placed in incubators to start treatment and the time that the samples were removed from the incubators and the carboxylic acids were washed from the *Ascaris* eggs (**Table A3.1-A3.4**).

5.4 Results and discussion

5.4.1 Experimental results

Of the 48 samples in the first CCD experiment, 27 of them contained no viable eggs and an additional 16 samples resulted in less than 1.0% viability (**Table A3.1, Figure 5.1A**). With only 5 samples representing 1-100% viability, we determined that any model built on this data alone would be inadequate. The second CCD experiment with reduced concentrations resulted in a greater range of responses with 19 out of 48 samples resulting in > 1% *Ascaris* viability (**Table A3.2, Figure 5.1B**). Group 3 and Group 4 were added because the observed viabilities in the second CCD were still dominated by low values (**Figure 5.1B**). Group 3 treatments primarily used the same factor levels that were used in the second CCD, but in combinations that were not included in the CCD and were expected to result in higher *Ascaris* viability. A lower exposure time of 3 days was also added, which fell between the CCD –α value of 1 day and the -1 value of 6 days. The responses for these samples were more evenly distributed than in Groups 1 and 2 (**Table A3.3, Figure 5.1C**). Group 4 was added to gain more data at higher carboxylic acid concentrations that are possible in real fermentation systems. Concentrations of 260 mM *n*-butyric acid, 12 mM *n*-valeric acid, and 27 mM *n*-caproic acid were used to match the highest concentrations reported previously in batch fermentation trials (Harroff et al., 2017). The concentrations were similar to those used for the first CCD. The exposure times and temperatures were reduced compared to the first CCD, but most Group 4 samples still resulted in no viable eggs (**Table A3.4, Figure 5.1D**).

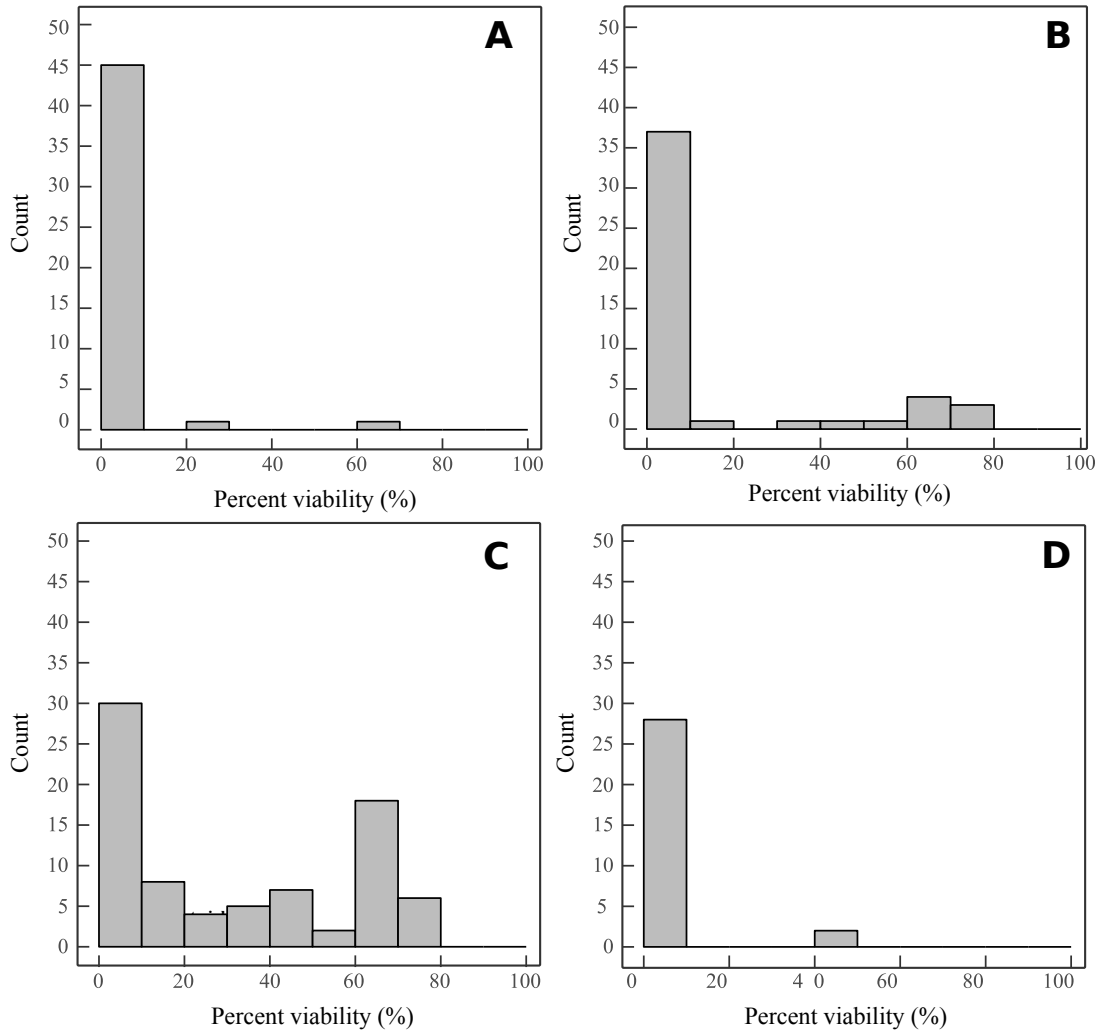

Figure 5.1 Histogram of responses for: (A) Group 1 (first CCD); (B) Group 2 (second CCD); (C) Group 3; and (D) Group 4.

5.4.2 Model developed using CCD provided a poor fit to independent data

We did not attempt to fit a model to the data from the first CCD experiment in Group 1 due to the low observed viability. For the second CCD experiment in Group 2, we fitted a second-order linear regression model (**Equation 5.1**), which is recommended for fitting data obtained from CCDs and contains all linear terms, quadratic terms, and two-way interactions (Oehlert, 2000).

$$Y = \beta_0 + \sum_{i=1}^{5} \beta_i X_i + \sum_{i=1}^{5} \beta_{ii} X_i^2 + \sum_{i=1}^{5} \sum_{j=i+1}^{5} \beta_{ij} X_i X_j \qquad \textbf{(Equation 5.1)}$$

Y is the response variable (percent viable), β_is are model coefficients, and X_is are predictor variables (*n*-butyric acid concentration, *n*-valeric acid concentration, *n*-caproic acid concentration, exposure time, and temperature) (**Table A3.5**). The adjusted coefficient of determination (R^2) was 0.834, indicating that 83.4% of the variation in the data was explained by the model, and a large global F statistic of 12.8 (df=20, 27, $p <$ 0.0001) indicated that the model was significant. One challenge with the linear regression equation is that our response variable (percent viability) is naturally bound between the values of 0 and 100, while the model can predict responses outside of this range. To address this discrepancy, we set any predicted responses greater than 100 as equal to 100, and any responses less than 0 as equal to 0. With this adjustment, the predicted and observed viabilities for the CCD data were highly correlated with R^2= 0.921 and a root mean square error (RMSE) of 7.55.

Overall, the model fit the experimental data well (**Figure 5.2A**), but it performed poorly when we tested it against independent results from Group 3 (**Figure 5.2B**). All factors in the Group 3 treatments were within the ranges used in the CCD, and thus the model should have accurately predicted the percent viability for these treatments. However, the RMSE for the Group 3 data was 19.4, which was substantially higher than for the CCD data, and the R^2 between predicted and observed viability was 0.782. We also compared the CCD model to the full dataset containing results from Groups 1-4, and it performed even worse, with RMSE=38.8 and R^2=0.380 (**Figure 5.2C**). The full dataset contained treatments outside the scope of the CCD data. Therefore, we were not surprised

to find that the model performed poorly here. However, a model that could predict *Ascaris* viability for the full range of Groups 1-4 was desired for practical purposes.

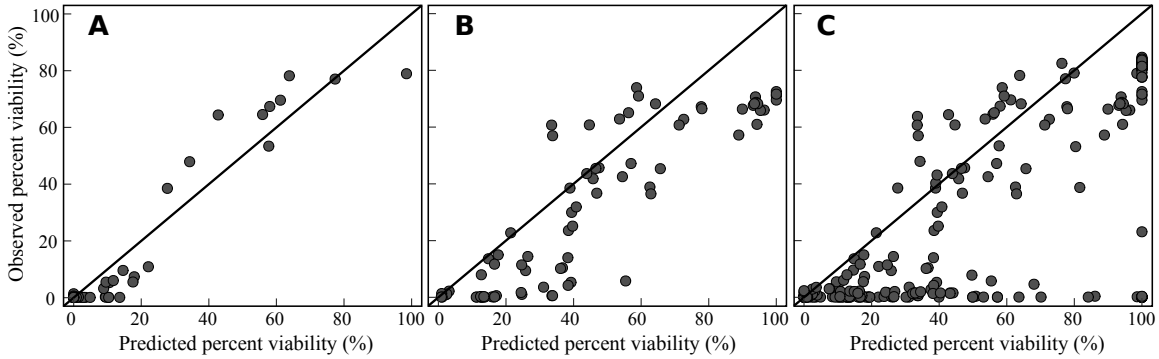

Figure 5.2 Observed and predicted percent viability for: (A) data from the second CCD experiment; (B) Group 3 data with all factors within the same ranges as the second CCD; and (C) all data from Groups 1-4. The model developed from the second CCD data (**Table A3.5**) was used for predicted viability.

The coefficients of the model were difficult to interpret because each factor was included six times: once as a linear term, once as a quadratic term, and four times as an interaction with every other factor. Instead, response surface plots are useful tools for visualizing the effects of each factor on viability. The response surfaces resulting from this model demonstrated poor performance at the upper regions of the CCD data, particularly between the coded +1 and +α values of the factors (i.e., *n*-butyric acid=165.0-260.0 mM, *n*-valeric acid=35.5-50.0 mM, *n*-caproic acid=9.9-14.0 mM, exposure time=13.8-19.0 d, and temperature=36.3-45.0°C) (**Figure 5.3**). In parts of these regions, the predicted percent viability increased as one of the variables increased. However, logically, *Ascaris* viability should only decrease as any of the variables increase. The unrealistic shapes of the response surfaces were caused by the quadratic

terms in the model, indicating that the form of the model was not appropriate to this type of data.

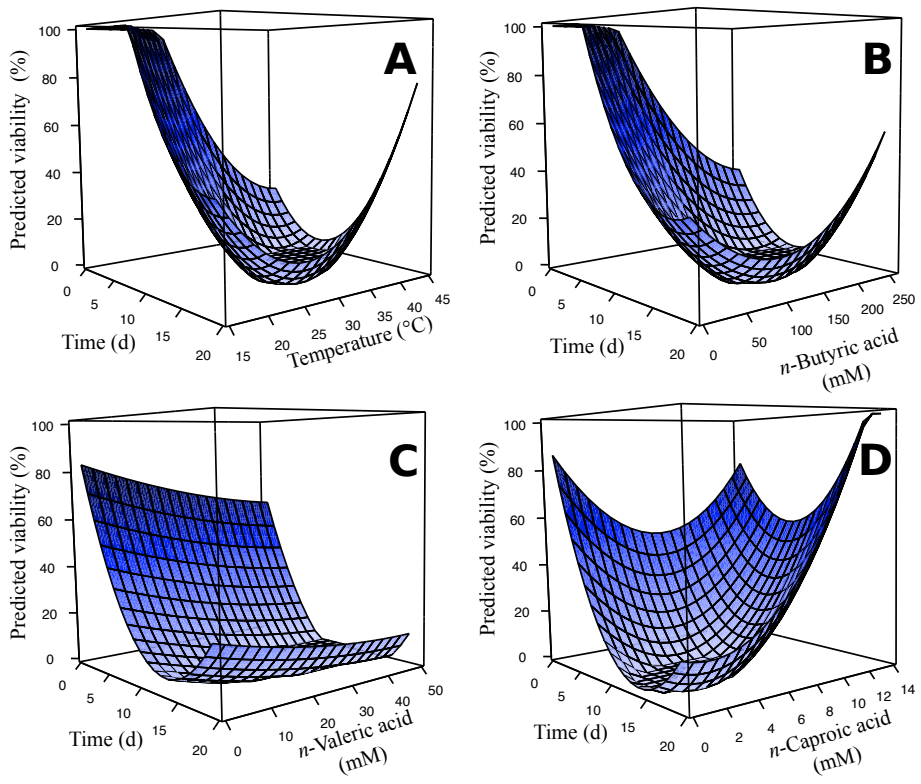

Figure 5.3 Response surface plots for linear regression model developed from the second CCD data using linear terms, quadratic terms, and two-way interactions for the five factors tested. Surfaces show predicted percent viability based on changes in exposure time and: (A) temperature; (B) *n*-butyric acid; (C) *n*-valeric acid; and (D) *n*-caproic acid. For each plot, any variables not shown are held constant at the center point of the second CCD (i.e., *n*-butyric acid=130.0 mM, *n*-valeric acid=25.0 mM, *n*-caproic acid=7.0 mM, and temperature =30.0°C).

5.4.3 Logistic regression provided a better predictive model.

Based on the unrealistic shapes of the response surfaces and the limited range of the CCD model, we used the full dataset from Groups 1-4 to develop and test new models using both linear and logistic regression. We suspected that logistic regression might be better suited for our data because logistic regression models are used to predict the probability

of discrete responses (e.g., viable or not viable) and are constrained between 0 and 1. We fitted both logistic and linear regression models to the data from Groups 1-4, using: (1) only linear terms of the tested factors; (2) linear terms and two-way interactions; (3) linear terms and quadratic terms; and (4) linear terms, quadratic terms, and two-way interactions. For the logistic regression models, we used the actual counts of viable and not viable *Ascaris* eggs for each sample as the response variable (**Tables A3.1-A3.4**), and for the linear regression models we used the proportion of viable eggs (percent viability divided by 100) as the response. This allowed us to compare the RMSE of each model with the same units. We compared the models using k-fold cross-validation repeated 100 times with k=10, and we found that for all four combinations of predictor terms, the logistic regression models performed better than the linear regression models (**Figure 5.4**). Of the logistic regression models, the one using only linear terms and two-way interactions (**Table 5.3**) was the best fit with a mean RMSE of 0.124 (**Figure 5.4**, **Figure A3.1**). The logistic regression model with linear terms, quadratic terms, and two-way interactions had a slightly lower mean RMSE of 0.122, but the modest decrease did not justify using a more complicated model. The variability in RMSE was also higher for the more complex model, indicating that the model may be less stable due to the extra predictors (**Figure 5.4**).

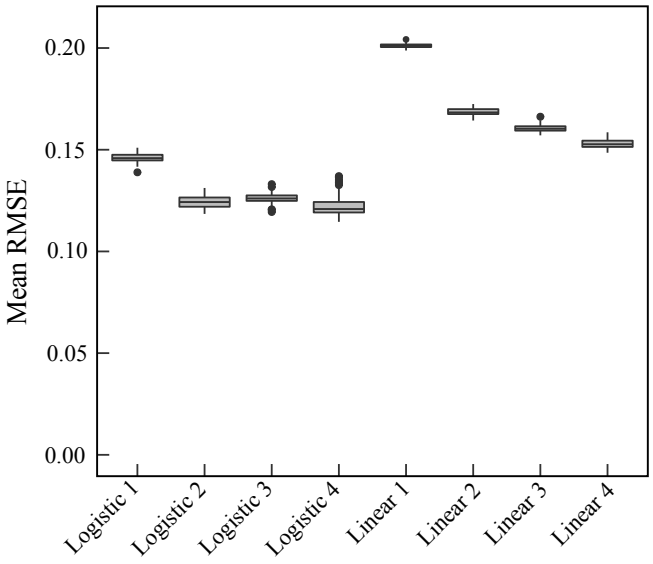

Figure 5.4 Boxplot showing mean root mean square error (RMSE) of the repeated k-fold cross-validation for each model tested. For both logistic and linear regression models, numbers indicate: (1) linear terms only; (2) linear terms and two-way interactions; (3) linear and quadratic terms; and (4) linear terms, quadratic terms, and two-way interactions.

Table 5.3 Coefficients and standard errors for logistic regression model containing linear terms and two-way interactions.

Predictor	Coefficient (log odds)	Standard error	*p*-value	Significance
Intercept	6.12	0.163	< 0.001	***
Bu	0.00214	0.00184	0.246	NS
Va	0.0916	0.00913	< 0.001	***
Ca	0.396	0.0292	< 0.001	***
Ti	-0.0239	0.0117	0.0405	*
Te	-0.151	0.00506	< 0.001	***
Bu ∗ Va	-7.45×10^{-5}	2.05×10^{-5}	< 0.001	***
Bu ∗ Ca	-7.72×10^{-4}	4.77×10^{-5}	< 0.001	***
Bu ∗ Ti	-0.00207	7.82×10^{-5}	< 0.001	***
Bu ∗ Te	-1.41×10^{-4}	5.75×10^{-5}	0.0143	*
Va ∗ Ca	-0.00195	2.31×10^{-4}	< 0.001	***
Va ∗ Ti	-0.00420	3.61×10^{-4}	< 0.001	***
Va ∗ Te	-0.00240	2.50×10^{-4}	< 0.001	***
Ca ∗ Ti	-0.0132	0.00117	< 0.001	***
Ca ∗ Te	-0.0116	8.44×10^{-4}	< 0.001	***
Ti ∗ Te	-4.90×10^{-4}	4.02×10^{-4}	0.223	NS

[a] *p*-value< 0.001 ***; *p*-value< 0.01 **; *p*-value< 0.05 *; *p*-value ≥0.05 NS

The shapes of the surface plots for the selected logistic regression model were also more reasonable than we observed for the previous CCD linear regression model, with the probability of an egg being viable decreasing when any of the five factors increased (**Figure 5.5**). By examining the shapes of the surfaces, we observed that both time and temperature had a strong effect on viability when carboxylic acids were present (**Figure 5.5A**). We observed a small shoulder at the shortest times and lowest temperatures, where an increase in either factor had little effect on viability. Beyond the shoulder, a small increase in either factor caused a sharp decrease in expected viability (**Figure 5.5A**). The surface for time and temperature shown here assumes that the carboxylic acid concentrations are equal to the center point of the second CCD (*n*-butyric acid=130.0 mM, *n*-valeric acid=25.0 mM, and *n*-caproic acid=7.0 mM). These are relatively low concentrations compared to what we expect from fermentation systems, and the time and temperature combinations required to achieve < 0.1% *Ascaris* viability are still reasonable, with about 15 days required at 30°C or 22 days required at 20°C (**Figure 5.5A**). When no carboxylic acids were present, the model predictions for inactivation based on time and temperature alone were conservative. Previous studies have found *Ascaris* inactivation after 14 days at 40°C (Pecson et al., 2007), after 6.2 days at 42°C (Senecal et al., 2018), and after less than 1 day at 45°C (Cruz Espinoza et al., 2012) when no additional inactivating agents were present. However, the model here predicts 11.5% viability after 30 days at 45°C when no carboxylic acids are present (**Figure A3.2**).

Of the carboxylic acids, *n*-butyric acid appeared to have the greatest effect on viability, but the different scales for each acid should be noted (**Figure 5.5B-5.5D**). When fermenting HFM, *n*-butyric acid is produced in higher concentrations than the other two acids. The larger concentrations cause it to have a greater effect on *Ascaris* viability, but the effect per mM increase in concentration is actually smaller than for *n*-valeric acid and *n*-caproic acid, which have longer hydrocarbon chains (**Figure A3.3**). The surfaces for *n*-valeric acid and *n*-caproic acid more clearly show the effect of chain length, with increasing *n*-caproic acid having a greater effect on viability than *n*-valeric acid, even with the larger range of concentrations for *n*-valeric acid (**Figure 5.5C-5.5D**). The greater efficacy of longer chain acids has been demonstrated previously for *Ascaris* eggs (Butkus et al., 2011; Harroff et al., 2017) and for other pathogens and bacteria (Abdul and Lloyd, 1985; Freese et al., 1973; Royce et al., 2013). Here we show that in fermentation systems, *n*-butyric acid remains critical to inactivation due to the larger concentrations produced, and *n*-valeric acid is likely not produced at high enough concentrations to greatly affect viability.

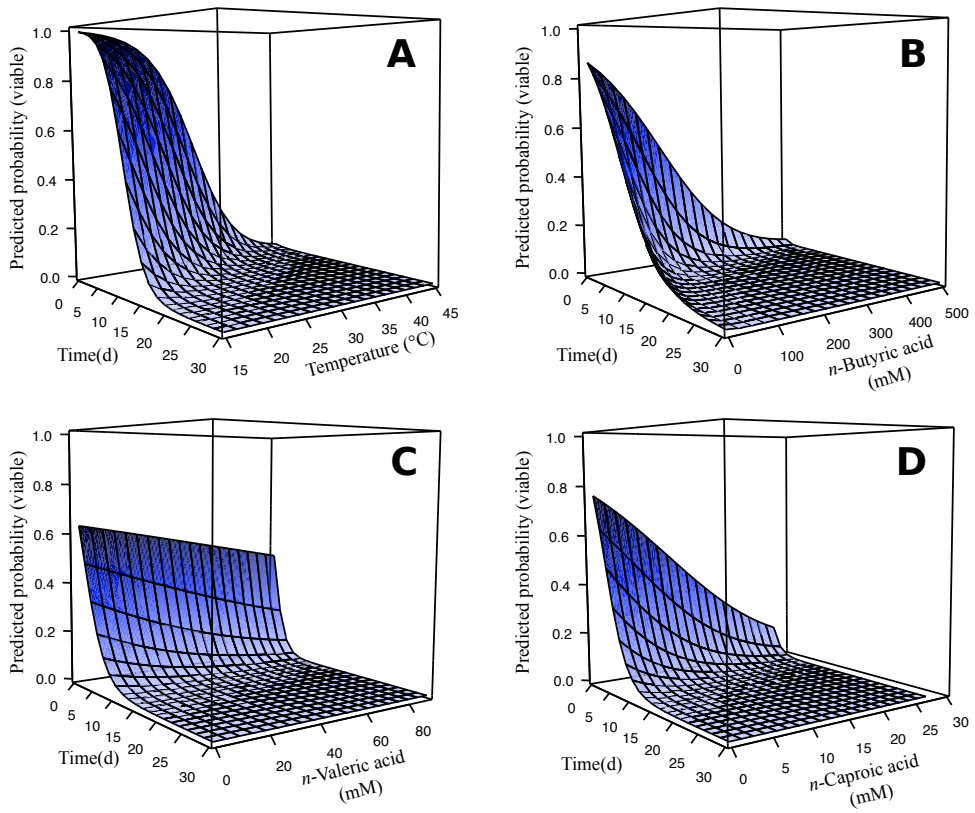

Figure 5.5 Response surface plots for the selected logistic regression model containing linear terms and two-way interactions for all five factors. Surfaces show the predicted probability of an *Ascaris* eggs being viable based on changes in exposure time and: (A) temperature, (B) *n*-butyric acid, (C) *n*-valeric acid, and (D) *n*-caproic acid. For each plot, any variables not shown are held constant at the center point of the second CCD (i.e., *n*-butyric acid=130.0 mM, *n*-valeric acid=25.0 mM, *n*-caproic acid=7.0 mM, and temperature=30.0°C).

5.4.4 Model over-predicted inactivation for Ascaris *eggs exposed to carboxylic acids in HFM matrix.*

Repeated k-fold cross-validation demonstrated that the model should be generalizable to an independent dataset, but there are few external datasets available regarding *Ascaris* inactivation by carboxylic acids, particularly using mixtures of multiple carboxylic acids. In one previously published experiment, HFM was spiked with *n*-butyric acid and *n*-caproic acid with pH adjusted to 4.7 to give average undissociated concentrations of 135.4 mM and 12.7 mM, respectively. Low levels of *n*-valeric acid were also naturally

present in the HFM, with an average undissociated concentration of 1.2 mM (Harroff et al., 2017). After 19 days at 30°C, *Ascaris* eggs remained 8.5% viable; however, the model developed here predicted that *Ascaris* eggs will be less than 0.1% viable after 14.5 days under the same conditions (**Table 5.4**). Observed experimental results from a similar treatment in the current study also demonstrated faster inactivation rates than were observed in the previous Harroff et al. study, indicating that the discrepancy may result from differences in the experimental data rather than from poor model fit. One of the samples tested in the second CCD contained 135.7 mM undissociated *n*-butyric acid, 0 mM *n*-valeric acid, and 6.8 mM undissociated *n*-caproic acid. It was incubated at 30.0°C for 9.8 days, and the resulting *Ascaris* viability was 5.5% (**Table 5.4, Table A3.2**). The predicted viability for this treatment was 4.5%, which is similar to the observed result.

Table 5.4 Comparison of observed and predicted *Ascaris* viability in similar treatments from a previously published study and the current study.

Study	Matrix	*n*-Butyric acid (mM)	*n*-Valeric acid (mM)	*n*-Caproic acid (mM)	Exposure time (d)	Temp (°C)	Observed viability (%)	Predicted viability (%)
Harroff et al., 2017	HFM [a]	135.4	1.2	12.7	19	30	8.5	< 0.1
Current	Aqueous solution	135.7	0	6.8	9.8	30	5.5	4.5

[a] Human fecal material

Although this is only a single data point, the result is surprising because we expected *Ascaris* inactivation observed in solutions to be conservative compared to inactivation in matrixes of HFM or manure slurry due to the presence of additional inactivating compounds (Nordin et al., 2009; Pecson et al., 2007; Schuh et al., 1985). However, non-aqueous matrices may be unevenly mixed, which causes pockets of poor inactivation (Jensen et al., 2009; Popat et al., 2010). Uneven distribution of carboxylic acids in the HFM may have caused slower inactivation in the previously published study

than we observed with aqueous matrices in the current study. Real treatment systems are also non-aqueous matrices. Therefore, our model may be unreliable for real treatment systems. Until it can be validated or improved with larger datasets, it should only be used to give an initial estimate of required treatment conditions for *Ascaris* inactivation, and actual inactivation should be verified with testing.

5.5 Conclusions

- A model was developed to predict *Ascaris* inactivation as a function of concentration of three carboxylic acids (*n*-butyric acid, *n*-valeric acid, and *n*-caproic acid), exposure time, and temperature. The model is applicable when each factor falls within the following ranges: (1) *n*-butyric acid=0-500 mM, (2) *n*-valeric acid=0-90 mM, (3) *n*-caproic acid=0-27 mM, (4) exposure time=0-30 d, and (5) temperature=15-45°C.
- Response surface methodology with a central composite design (CCD) was initially used for experimental design, but the limited number of treatments used in CCD was not adequate to capture the effects of all five inactivating factors.
- A logistic regression model using data from Groups 1-4 performed better than a linear regression model due to the proportional nature of the response variable (percent viability). The average RMSE for the selected model using repeated k-fold cross validation with k=10 was 0.124, indicating that the model fit the data well and can be generalized to independent datasets.
- Small increases in time and temperature caused large increases in inactivation rates.
- *n*-Butyric acid and *n*-caproic acid both heavily influence inactivation. The large impact of *n*-butyric acid is primarily due to the higher concentrations observed in

fermentation systems, while *n*-caproic acid has greater impact per unit increase of concentration. At the concentrations observed in fermentation systems, *n*-valeric acid has a minimal effect on *Ascaris* inactivation.

- Predicted combinations of carboxylic acid concentrations, exposure times, and temperature required for > 99.9% inactivation of *Ascaris* eggs are reasonable for real waste treatment systems. Concentrations are within the range of concentrations that have previously been produced by fermentation, and inactivation at ambient temperatures near 20°C and with exposure times less than 25 days are possible.
- In an external study, inactivation of *Ascaris* eggs by carboxylic acids in HFM was slower than predicted by the model developed here. This indicates that inactivation in real matrices might be slower than predicted using data from aqueous matrices. Until the model can be validated or improved with additional data, testing will be required to verify *Ascaris* inactivation in real treatment systems.

5.6 Acknowledgements

LH thanks Lucinda Li for assistance in determining *Ascaris* egg viability and acknowledges support from the Cross-Scale Biogeochemistry and Climate NSF IGERT program (Award #1069193) and the NSF Graduate Research Fellowship Program (Award #1650441). LTA acknowledges support from the Alexander von Humboldt Foundation in the framework of the Alexander von Humboldt Professorship endowed by the Federal Ministry of Education and Research in Germany.

5.7 Supporting information

Supporting information is provided in Appendix 3. It contains three figures and five tables containing data from individual treatments, model coefficients for the CCD model, and figures used to visualize model fit and interpretation.

CHAPTER 6

FIELD-SCALE CO-FERMENTATION OF SOLID WASTE FROM URINE-DIVERTING DRY TOILETS (UDDT-SW) AND BANANA WASTE TO PRODUCE UNDISSOCIATED CARBOXYLIC ACIDS TO INACTIVATE *ASCARIS* EGGS

Adapted from: Harroff, L.A., Liotta, J.L., Wangolo, E.E., Egan, T.J.S., Bowman, D.D., and Angenent, L.T. In preparation. Field-scale co-fermentation of solid waste from urine-diverting dry toilets (UUDT-SW) and banana waste to produce undissociated carboxylic acids to inactivate *Ascaris* eggs. In preparation for *Frontiers in Environmental Science*.

6.1 Abstract

Innovative, low-cost methods for inactivating pathogens in human fecal material (HFM) are needed, particularly in expanding urban areas where conventional sewer systems and centralized wastewater treatment plants are not feasible. To address this challenge, we have developed a bioprocess that utilizes open cultures of anaerobic bacteria (i.e., microbiomes) to produce carboxylic acids using HFM as substrate. When the pH is sufficiently low, the carboxylic acids exist in the undissociated form and inactivate pathogens. Here, we used real sanitation waste collected from container-based, urine-diverting dry toilets (UDDT-SW) in Nairobi, Kenya to conduct lab-scale and field-scale trials. Through these trials, we investigated operating conditions required to use carboxylic acid fermentation in sanitation waste treatment processes. We tested three different inoculum treatments and determined that the microbiome in UDDT-SW is well suited to produce carboxylic acids without the need for an external inoculum. We also

tested co-fermentation of UDDT-SW with carbohydrate-rich food waste as a means of reducing the pH. We found that when food waste was incorporated in a way that maintained the pH between 4.8 and 5.2, then the food waste was quickly converted to carboxylic acids, and the low pH created high concentrations of undissociated carboxylic acids. The resulting concentrations of undissociated carboxylic acids resulted in *Ascaris* inactivation within 15 days. However, we found that a temperature ≥30°C is required for carboxylic acid production to occur.

6.2 Introduction

As a global community, we struggle to manage our waste in a safe and sustainable manner. Management of human excreta is particularly concerning due to the implications it has for human health and dignity, and yet 4.5 billion people still lack access to safely managed sanitation services (WHO and UNICEF, 2017). The provision of a sanitation facility alone does not address the challenge because excreta must also be either safely contained on-site or transported, and treated off-site. Connection to a sewer system also does not guarantee access to safely managed sanitation. Of the 2.8 billion people using sewer connections, only 1.9 billion people are expected to have safely managed sanitation due to sewer lines that empty directly into the environment or wastewater treatment plants with insufficient treatment (WHO and UNICEF, 2017). Sewer systems are also frequently unfeasible due to high costs, uncertain land tenure, lack of appropriate space, and challenging topography among others (World Bank, 2019). Instead, a variety of treatment approaches and technologies are required to address the diverse challenges

faced in achieving universal safe sanitation, including innovative methods for inactivating pathogens in excreta.

One method under investigation uses anaerobic fermentation of human fecal material (HFM) to produce carboxylic acids (Harroff et al., 2017). Complex organic wastes are first broken down to short-chain carboxylic acids (e.g., acetic acid, propionic acid, and *n*-butyric acid) through hydrolysis and primary fermentation. Oxidation of an electron donor, such as ethanol or lactate, is then coupled to chain elongation of the short-chain carboxylic acids to produce *n*-butyric acid (C4), *n*-caproic acid (C6), and even *n*-caprylic acid (C8) (Agler et al., 2011; Spirito et al., 2014). Acetic acid (C2) is often the dominant primary fermentation product, and hydrocarbon chains are elongated with two carbon molecules at a time. Therefore, molecules with even-numbered hydrocarbon chains are more common than those with odd-numbered chains, but both are produced. Previous work has shown that HFM can be used as substrate to produce up to 257 mM *n*-butyric acid, 11.3 mM *n*-valeric acid, and 27.1 mM *n*-caproic acid (Harroff et al., 2017).

Carboxylic acids exist in both a dissociated (carboxylate) and undissociated (carboxylic acid) form, depending on the pH in the solution. When the pH is below the pKa (~4.8 for the carboxylic acids of interest here), a majority of the molecules are in the undissociated form with a hydrogen atom attached to the carboxyl group. At a pH above the pKa, a majority of the molecules exist as the dissociated carboxylate with the hydrogen detached. The specific concentrations can be calculated using the Henderson-Hasselbalch equation, $pH = pK_a + log\,([A^-]/[HA])$, where [$A^-$] represents the concentration of the dissociated form, and [HA] represents the concentration of the undissociated form. Throughout this paper, we will explicitly specify if we are referring

to the undissociated form. If the undissociated form is not specified, then we are referring to the total concentration of undissociated acid plus dissociated conjugate base.

The distinction between the total concentration and the concentration of undissociated carboxylic acids is important because only the undissociated form inhibits pathogens (Butkus et al., 2011; Harroff et al., 2017; Royce et al., 2013). We previously produced carboxylic acids from HFM, but the pH remained nearly neutral throughout the experiment. Therefore, the fraction of undissociated carboxylic acids form remained relatively low, while the total carboxylic acid concentrations were promising (Harroff et al., 2017). Co-fermentation of HFM and food waste may present a solution to this problem. Many studies have shown that anaerobic fermentation or co-fermentation of carbohydrate-rich food waste results in rapid lactic acid production and pH decline (Liang and Wan, 2015; Rajagopal et al., 2014; Wang et al., 2014). For one study, workers co-fermented mixed food waste and HFM specifically to increase concentrations of undissociated carboxylic acids to inactivate pathogens, but this study did not focus on chain elongation (Riungu et al., 2018). However, co-fermentation with food waste may be challenging because low pH and increased undissociated carboxylic acid concentrations can inhibit the bacteria responsible for fermentation and chain elongation, in addition to inactivating pathogens (Agler et al., 2012; Riungu et al., 2018). Therefore, pH reduction must be carefully balanced with maximizing carboxylic acid production.

The primary goal of our study was to better understand how HFM can be fermented to produce undissociated carboxylic acids that cause pathogen inactivation in real sanitation and waste treatment systems. To accomplish this, we collaborated with Sanergy, which is a sanitation enterprise in Nairobi, Kenya, that maintains a network of container-based,

urine-diverting dry toilets (UDDTs) and converts the collected waste to valuable reuse products. Carboxylic acid fermentation may be useful as a pretreatment step in treatment facilities similar to Sanergy's facility, because the output has an adequately reduced pathogen load and has already undergone the often rate-limiting stages of biological degradation. It can then be used as the input for further processing into value-added products such as biogas from anaerobic digestion or livestock feed from black soldier fly larvae production. Here, we used real HFM collected from Sanergy's UDDTs and tested fermentation: (1) with 500-mL media bottles in the lab during Experiments 1 and 2; and (2) 45-L plastic barrels at the field-scale during Experiment 3. We tested three different inocula to determine if a specialized inoculum would be required to implement new treatment systems. We also tested the addition of banana waste at different times during the fermentation process and at different ratio to maximize concentrations of undissociated carboxylic acids. Finally, we demonstrated pathogen inactivation from co-fermentation of HFM with banana waste using *Ascaris suum* eggs as indicators.

6.3 Materials

6.3.1 HFM samples

HFM was obtained from UDDTs that are located in informal settlements around Nairobi, Kenya. The UDDTs have separate containers to collect urine and solid waste. The UDDT solid waste (UDDT-SW) container is 30 L and contains a mixture of HFM and sawdust, which is added by users to reduce odor in the toilets. Sawdust is expected to constitute about 20% of the total mass of the UDDT-SW. Sanergy collects the urine and UDDT-SW containers each day and replaces them with clean containers. On the starting day for

Experiments 1 and 3, samples of UDDT-SW were taken from several containers that had been collected from UDDTs that same day.

6.3.2 Banana waste

The pre-consumer banana waste originated from Twiga Foods, which is a food supply platform that connects smallholder farms to market vendors. Sanergy receives fresh banana waste that is unsuitable for market every few days and stores it outdoors in a pile until it is used in waste treatment and recycling processes.

6.3.3 Chain-elongation-bioreactor inoculum

The chain-elongation-bioreactor inoculum originated from a semi-continuous lab-scale bioreactor that chain-elongated ethanol-rich fermentation beer for 4 years (Ge et al., 2015). The effluent was collected from the bioreactor and centrifuged to concentrate the biomass. The bioreactor broth was discarded, and the biomass was re-suspended in an equivalent volume of liquid that was 50% (v/v) glycerol and 50% (v/v) fresh bioreactor media. The biomass was then frozen at -80°C for approximately one year until use. After thawing, the biomass was washed twice to remove the glycerol by centrifuging and replacing the supernatant with tap water. A new culture was then started by inoculating 10% (m/m) biomass into diluted UDDT-SW. This culture was kept in an airtight 1-L glass bottle. The headspace was sparged with nitrogen gas to create anaerobic conditions, and the bottle was incubated at 30°C in the dark for 24 days with periodic manual shaking to mix. Contents from this culture were used directly as chain-elongation-bioreactor inoculum for lab-scale experiments.

6.3.4 Rumen inoculum

Rumen contents were selected for testing because significant carboxylic acid production occurs in the rumen, and rumen contents were originally used to inoculate the bioreactor that was used for the chain-elongation-bioreactor inoculum (Agler et al., 2012). The rumen contents are also easier to obtain than chain-elongation-bioreactor inoculum, which would assist in successful implementation of carboxylic acid fermentation for waste treatment. The rumen inoculum was collected from the rumen contents of a cow that had been freshly slaughtered at Dagoretti Slaughterhouse in Nairobi, Kenya. Next, the rumen contents were strained through a mesh screen and cheesecloth to remove large particulate matter before use.

6.4 Methods

6.4.1 Experiment 1: Determining the importance of a specialized inoculum

Three inoculum treatments were tested to determine whether a specialized inoculum was necessary to achieve carboxylic acid production and chain elongation: (1) chain-elongation-bioreactor inoculum; (2) rumen inoculum; and (3) no inoculum. The chain-elongation-bioreactor inoculum and rumen inoculum were obtained as described above. For the third treatment, tap water was used in place of an inoculum, and therefore the fermentation in the media bottles for this treatment was dependent on the microbiome residing naturally in HFM. UDDT-SW was collected in the morning of the starting day of the experiment. The UDDT-SW samples were combined and homogenized in a kitchen

blender before separating into treatments. Each treatment was prepared with approximately 65 g inoculum and 585 g UDDT-SW (**Table 6.1**). The treatment mixtures were manually mixed with a spatula, and 200 g was allocated to triplicate 500-mL glass media bottles. A 5-g sample of each treatment was also kept for pH and carboxylic acid analysis. The headspace in each media bottle was sparged with nitrogen gas for 1 min at a rate of 12 L min^{-1}. The media bottles were then sealed with a rubber stopper and plastic ring (**Figure A4.1**) and incubated in the dark at 30°C for 26 days. Additional mixing occurred only during sample collection. The fermentation gas was vented from the media bottles on Day 1 and 2 to avoid excessive pressure buildup before the first sampling point. Venting was performed by inserting a hypodermic needle into a septum that was located in the rubber stopper of each media bottle. The samples were collected from each media bottle on Day 5, 12, 18, 21, 25, and 26 to measure pH and carboxylic acid concentrations. The rubber stopper and plastic ring were removed from the media bottle, and the contents were manually mixed with a spatula before removing a small sample (~5 g) of contents. Finally, the headspace in the media bottle was then sparged with nitrogen for 1 min at 12 L min^{-1}, and the rubber stopper and plastic ring were replaced. Carboxylic acid concentrations were measured using gas chromatography (Hewlett-Packard 5890 Series II; Wilmington, DE) with a method described previously, except nitrogen was used as the carrier gas instead of helium (Usack and Angenent, 2015).

Table 6.1 Initial conditions for Experiment 1. Masses are total mass for the triplicate media bottles for each treatment.

Inoculum type	Mass inoculum (g)	Mass HFM (g)	% Inoculum (m/m)	Moisture content (%)
1: Chain-elongation-bioreactor	65.4	584.9	10.1	84.0
2: Rumen	65.1	587.1	10.0	83.7
3: None (tap water)	65.3	586.1	10.0	84.7

6.4.2 Experiment 2: Co-fermentation with banana waste to reduce pH, increase concentrations of undissociated carboxylic acids, and inactivate Ascaris *eggs*

After Day 26 of the operating period, the media bottles from Experiment 1 were emptied. All of the remaining fermented UDDT-SW was combined, homogenized in a kitchen blender, and divided into three new treatments: (1) 0% banana-mash addition; (2) 10% banana-mash addition; and (3) 20% banana-mash addition, with all percentages on a mass basis. The banana waste had been collected from the outdoor storage pile one day prior to Day 0 and kept in the laboratory refrigerator overnight. The peels from the bananas were removed, and the bananas were homogenized in a kitchen blender. Finally, the resulting banana mash was combined with the fermented UDDT-SW to produce 360 g of each treatment. The mixtures were manually mixed with a spatula, and 155-g aliquots were distributed to duplicate 500-mL glass media bottles. We used duplicate treatments instead of triplicates based on limitations from the quantity of fermented UDDT-SW material available. The media bottles (**Figure A4.1**) were sparged with nitrogen, sealed, and sampled as described above, with samples collected on Day 1, 3, 6, and 15 to measure pH and carboxylic acid concentrations.

We tested *Ascaris* inactivation for the 0% and 20% banana-mash treatments using *A. suum* eggs. The eggs were collected from fecal material in the intestines of naturally infected slaughterhouse pigs and purified and stored as described previously (Harroff et al., 2017). We used plastic chambers (2.5 cm diameter, 2.5 cm height), which were sealed on both ends with 38-µm metal mesh, to contain the eggs while exposing them to the conditions inside the media bottles. The plastic chambers were initially sealed on one

end, and the open end was used to fill them with the 0% and 20% banana-mash treatment mixtures and to spike them with 10,000 unembryonated *A. suum* eggs. A soldering iron was then used to seal the open end of the plastic chamber with a second piece of mesh (**Figure A4.2**). Nylon mesh bags have frequently been used to contain *Ascaris* eggs in similar experiments (Fidjeland et al., 2016; Johnson et al., 1998; Manser et al., 2015; Nordin et al., 2009), but we found that the plastic chambers were more reliable. Prior to performing the experiment, we made several nylon mesh bags and sealed the seams with a thermal sealer, as has been done before, but we found that the seams separated over time or had incomplete seals. In a study that directly compared *Ascaris* egg recovery from nylon mesh bags versus plastic chambers, plastic chambers were also found to perform better and more consistently (Nelson and Darby, 2002). The mesh bags often trapped gas inside, and the seams became unsealed, possibly due to the increased gas pressure. In contrast, the fixed volume of the plastic chambers seemed to force gas out of the chamber, and the more substantial seal held throughout the experiment (Nelson and Darby, 2002).

Eight plastic chambers were prepared, with two plastic chambers placed in each of the duplicate media bottles for the 0% and 20% banana-mash treatments. One plastic chamber from each media bottle was removed during sampling on Day 6 and 15. *Ascaris* eggs were recovered from the plastic chambers using a method adapted from several methods published previously (Amoah et al., 2018; Bowman et al., 2003; Nelson and Darby, 2002; Trönnberg et al., 2010). First, the mesh from one side was peeled off, and a metal spatula was used to divide most of the contents between two 50-mL centrifuge tubes. The empty plastic chamber was then placed in a 200-mL wide-mouth cylinder with

about 75 mL of 1.5 M ammonium bicarbonate (AmBic) solution. The cylinder was sealed and shaken for 30 s to dislodge any remaining solid material. The solution in the cylinder was poured into the two centrifuge tubes, and additional AmBic solution was used to rinse the chamber and cylinder into the tubes. The two centrifuge tubes were sealed and shaken for 1 min and then centrifuged at 1,000 × g for 5 min. The supernatant was discarded, and the tubes were filled with $MgSO_4$ (specific gravity = 1.2), vortexed, and centrifuged again at 1,000 × g for 5 min to float the *Ascaris* eggs. The supernatant was sieved through US 50 and US 200 sieves, before collecting the eggs on a US 400 sieve. Diluted H_2SO_4 (0.1 N) was used to rinse the sieve into a clean 50-mL centrifuge tube. The tube was centrifuged at 1,000 × g for 5 min to concentrate the eggs at the bottom of the tube, and a pipette was used to remove supernatant to a final volume of 5 mL. Next, the tube was vortexed for a final time, the cap was loosened to allow oxygen flow, and the tube was incubated in the dark at 28°C for 3 weeks to allow any viable *A. suum* eggs to develop into larvae. After 3 weeks, at least 100 eggs were observed microscopically to determine viability. Fully developed larvae were considered viable, and all others were not.

6.4.3 Experiment 3: Field-scale trial

A field-scale fermentation trial was performed on-site at Sanergy's waste processing facility located southeast of central Nairobi in Kinanie. Six 45-L screw-top plastic barrels were used as batch bioreactors to test three treatments in duplicate (**Table 6.2, Figure A4.3**). For Treatments 1 and 2, UDDT-SW was fermented alone for 42 days before adding banana waste because we expected the banana waste to cause a low pH that would

be inhibitory to carboxylic acid production. For Treatment 3, banana waste was mixed with UDDT-SW on Day 0, and no additional banana waste was added on Day 42 of the operating periods. We used cow rumen inoculum for Treatments 1 and 3, and no inoculum for Treatment 2. We obtained rumen contents from a cow that was slaughtered the same morning, and we prepared the inoculum as described above. UDDT-SW was collected from two randomly selected storage containers that had been transferred to Kinanie within the previous 2-3 days. Each storage container held UDDT-SW collected from approximately 3-4 UDDTs. We collected banana waste from the storage pile onsite and roughly chopped the waste with the peels left intact. The required material for each barrel was combined and manually mixed with a shovel.

Data loggers (HOBO UX100-003, Bourne, MA and Lascar Electronics EL-USB-1, Eerie, PA) were contained in sealed plastic tubes and placed in each barrel to record temperature every 15 min. The barrels were initially placed in a sunny area outside and covered with a black tarp to promote warmer temperatures. We conducted the field trial in May-July, which is the cool season for Nairobi. After 29 days of operation, the barrels were moved inside a greenhouse to achieve warmer temperatures. Throughout the operating period, samples were collected every 2-6 days from the barrels. At sampling, the lid was removed, and the barrel contents were manually mixed with a large spoon. Small samples were collected from several points in the barrel and mixed together in a plastic tube. The composite sample was used to measure pH and carboxylic acid concentrations.

Table 6.2 Treatment summary for field-scale trial (Experiment 3). Masses given are per barrel, with duplicate barrels used for each treatment.

Treatment #	Initial setup				Second stage (Day 42)
	UDDT-SW (kg)	Inoculum type	Inoculum mass (kg)	Banana waste (kg)	Banana waste (kg)
1	22.5	Rumen	2.5	0	6.5
2	22.5	None (tap water)	2.5	0	6.5
3	17.5	Rumen	2.5	5	0

We also tested *Ascaris* inactivation in the field-scale trial. First, we placed *A. suum* eggs in plastic chambers as described above. Ten chambers were prepared for each barrel, with two chambers collected from each barrel at each time point. The chambers were attached to wires and sunk in the barrels to expose the *A. suum* eggs to the fermentation conditions. An additional 20 chambers were prepared and filled with autoclaved UDDT-SW instead of the UDDT-SW used in the barrels. Each of these chambers was sealed in a 50-mL plastic tube that was also filled with autoclaved UDDT-SW, and the plastic tubes were sunk in the barrels for Treatment 1. Duplicate chambers with the autoclaved UDDT-SW were collected at the same time points as the other chambers and were used to isolate inactivation that occurred for reasons unrelated to the fermentation process (e.g., temperature or compounds already existing in the UDDT-SW before fermentation).

6.5 Results and discussion

6.5.1 Experiment 1: Specialized inoculum was not necessary to produce carboxylic acids in HFM

During 26 days of fermentation for the lab-scale trial, we found no benefit from either the chain-elongation-bioreactor inoculum or rumen inoculum compared to the no-

inoculum treatment (**Figure 6.1A and 6.1B**). The *n*-butyric acid concentrations between the three treatments were nearly identical throughout the operating period with maximum average concentrations observed on Day 25 for all three treatments (**Figure 6.1A**). The average maximum *n*-butyric acid concentrations were 107 mM, 111 mM, and 107 mM for chain-elongation-bioreactor inoculum, rumen inoculum, and no inoculum, respectively, but the differences were within the standard deviations of the triplicate samples (**Figure 6.1A**). For *n*-caproic acid, the treatment with no inoculum actually outperformed the other two treatments with an average maximum concentration of 24.3 mM versus 21.0 mM with chain-elongation-bioreactor inoculum and 20.0 mM with rumen inoculum (**Figure 6.1B**). The *n*-caproate concentration for the chain-elongation-bioreactor inoculum treatment consistently lagged behind the other two treatments for the first 18 days of the operating period (**Figure 6.1B**). We also observed that the trends for *n*-valeric acid concentrations for the batches were similar to the *n*-caproic acid concentrations (**Figure A4.4**). The treatment with no inoculum consistently produced the highest *n*-valeric acid concentrations, and a maximum average *n*-valeric acid concentration of 35.0 mM was observed on Day 25 of the operating period (**Figure A4.4**). This *n*-valeric acid concentration is notably larger than the 11.3 mM observed in a previously published study (Harroff et al., 2017) and may be the result of competing pathways before entry into the chain-elongating pathway. Such pathways include the acrylate pathway, which converts lactic acid into propionic acid (Kucek et al., 2016). Chain elongation of propionic acid would then result in production of *n*-valeric acid instead of one of the even-numbered carboxylic acids. Although we did not measure lactic acid here, it is a common product of primary fermentation in addition to acetic acid,

and is quickly removed while being produced in the anaerobic food web. We observed only a small difference in pH between the three treatments and throughout the time-course of the experiment. The average pH was 6.2 with a minimum of 6.0 and maximum of 6.3 (data not shown), which is similar to the previously published study (Harroff et al., 2017).

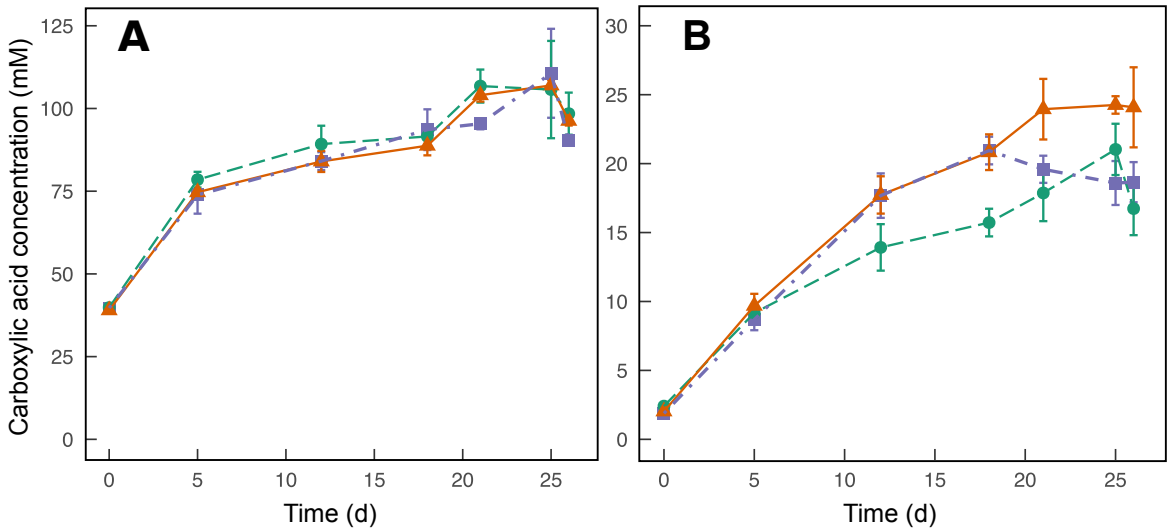

Figure 6.1 Average concentrations of *n*-butyric acid (A) and *n*-caproic acid (B) found in triplicate batch fermentation media bottles that were started with inoculum from a semi-continuous chain-elongation bioreactor (●); inoculum from rumen contents of a cow (■); and no inoculum (▲). Error bars indicate standard deviations of the measured concentrations.

The promising performance of the treatment with no inoculum indicates that the gut microbiome, which is inherent to HFM, can be used to produce carboxylic acids. This finding is not surprising because the human-gut microbiome is already known to produce *n*-butyric acid and *n*-caproic acid within the digestive system (Cummings et al., 1987; Miller and Wolin, 1996). While not surprising, the finding is important for implementing the proposed treatment method in sanitation systems because the system becomes less complex and less expensive if a specialized inoculum is not required. However, human

diet is known to affect the gut microbial community and resulting fermentation products (Flint et al., 2015). Therefore, variability in rates of carboxylic acid production and final concentrations should be expected when relying on the gut microbiome.

6.5.2 Experiment 2: Co-fermentation with banana mash and Ascaris *inactivation*

Co-fermentation with banana mash reduces pH

We observed only small changes in pH throughout the operating period when solely UDDT-SW was fermented for Experiment 1 (average pH of 6.2), which continued during Experiment 2 for the 0% banana-mash treatment (**Figure 6.2A**). A pH value of 6.2 causes only 4.2% of the total carboxylic acid concentration to exist in the undissociated form (**Figure 6.2B-C, A4.5, A4.6**). After banana mash was mixed with the fermented UDDT-SW at a rate of 20% by mass, the pH dropped from 6.1 to 4.8 within 6 days of the operating period (**Figure 6.2A**). At such a pH value, half of the total carboxylic acid concentration was in the undissociated form (**Figure 6.2B-C, A4.5, A4.6**). In the 10% banana-mash treatment, the pH decreased to 5.2 within 6 days (**Figure 6.2A**), resulting in 32% of the total carboxylic acid concentration in the undissociated form (**Figure 6.2B-C, A4.5, A5.6**). A previous study co-fermented mixed food waste and UDDT-SW at a series of substrate ratios and observed similar pH values compared to our study (Riungu et al., 2018). When food waste made up 20% of the total mass, the pH decreased from 5.7 to 4.9 in 4 days (Riungu et al., 2018).

Low pH causes inhibition of carboxylic acid production

On Day 0 for Experiment 2, carboxylic acid concentrations were already 73.5-88.7 mM *n*-butyric acid, 23.3-27.3 mM *n*-valeric acid, and 15.4-17.1 mM *n*-caproic acid due the pre-fermentation during Experiment 1. The addition of banana mash lowered these concentrations slightly due to dilution (**Figure 6.2B, A4.5A, and A4.6A**). When no banana mash was added, carboxylic acid concentrations continued to increase for the first three days of the operating period before tapering off (**Figure 6.2B, A4.5A, and A4.6A**). This result is similar to the result we would have anticipated if we had allowed the Experiment 1 treatments to continue fermenting. We observed maximum average concentrations for the 0% banana-mash treatment of 101 mM *n*-butyric acid, 31.4 mM *n*-valeric acid, and 21.7 mM *n*-caproic acid. The *n*-caproic acid concentration was similar to the maximum average concentration we observed in a previous fermentation experiment, but the *n*-butyric acid concentration was less than half of the concentration we observed (Harroff et al., 2017). Similar to Experiment 1, concentrations of *n*-valeric acid were higher than those seen previously (**Figure A4.6**), and this may partly account for the lower concentrations of *n*-butyric acid. In addition, a higher conversion efficiency of *n*-butyric acid to *n*-caproic acid may also have contributed to a relatively low *n*-butyric acid concentration.

For the 20% banana-mash treatment, no additional carboxylic acid production occurred (**Figure 6.2B, A4.5A, A4.6A**), which can be attributed to a combination of inhibition from: (1) the low pH directly; and (2) the high concentration of undissociated carboxylic acids, which was considerably increased due to the low pH (**Figure 6.2C, A4.5B, A4.6B)** (Agler et al., 2012; Riungu et al., 2018). For the 10% banana-mash

treatment, production rates of carboxylic acids were slightly higher than those observed for the 0% banana-mash treatment (**Figure 6.2B, A4.5A, A4.6A**). While the pH declined for the 10% banana-mash treatment, it remained above 5 (**Figure 6.2A**), which did not cause noticeable inhibition compared to the 0% banana-mash treatment. Therefore, the added banana mash provided a readily degradable carbon course that contributed to the increased production rates of carboxylic acids compared to the 0% banana-mash treatment.

Low pH must be balanced with carboxylic acid production to produce high concentrations of undissociated carboxylic acids

Although no additional carboxylic acid production occurred after banana mash was added in the 20% treatment, this treatment still resulted in the highest concentrations of undissociated carboxylic acids due to the lower pH value compared to the 0% and 10% treatments (**Figure 6.2C, A4.5B, A4.6B**). The difference in *n*-caproic acid concentrations between the 20% treatment and the 10% treatment was minor (**Figure 6.2C**), but it was more pronounced for *n*-valeric acid and *n*-butyric acid concentrations **(Figure A4.5B and A4.6B**). The concentration of undissociated carboxylic acids is a function of the total carboxylic acid concentration and the pH value. The pH relationship is exponential and has a greater effect than the total carboxylic acid concentrations. This is clearly visualized by the much higher concentrations of undissociated *n*-caproic acid in the 10% and 20% banana-mash treatments compared to the 0% banana-mash treatment, while the total concentrations of *n*-caproic acid were somewhat equivalent between the three treatments (**Figure 6.2B and 6.2C**). Accordingly, the higher the total concentration, the higher the

undissociated concentrations can be achieved. Therefore, the baseline concentrations of carboxylic acids produced during Experiment 1 were important during Experiment 2. Without it, the 20% treatment would have had very low total carboxylic acid concentrations (and therefore low undissociated concentrations) due to inhibition from low pH after banana-mash addition. For 20% banana-mash addition, the two-stage fermentation is appropriate, which we performed here. However, it would have been simpler to conduct the fermentation in a single stage when UDDT-SW and banana waste are mixed upfront. For this scenario to be successful, a lower fraction of banana waste would have maintained a moderate pH (~5), which we observed for the 10% banana-mash treatment, from the beginning of the operating period, which would not have inhibited carboxylic acid production.

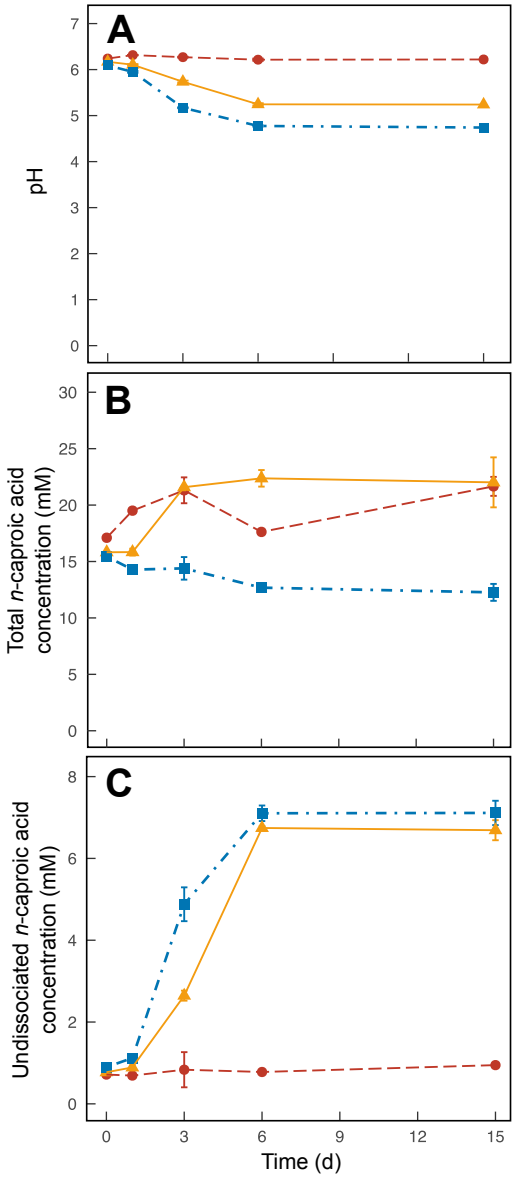

Figure 6.2 Average pH (A), total *n*-caproic acid concentration (undissociated plus dissociated) (B), and undissociated *n*-caproic acid concentration (C) found in duplicate batch fermentation media bottles with banana mash added at 0 % (m/m) (●), 10 % (m/m) (▲), and 20 % (m/m) (■). Error bars indicate the range of the measured values between the duplicate media bottles.

***Ascaris* eggs are inactivated faster when banana mash is used to produce a higher concentration of undissociated carboxylic acids**

We only compared *Ascaris* egg inactivation for the treatments containing 20% banana mash and 0% banana mash. For both treatments, we observed large decreases in *Ascaris* viability after 6 days and 15 days, but viability below detection was only achieved for the 20% banana-mash treatment after 15 days (**Figure 6.3**). The inactivation rates were also faster for the 20% banana-mash treatment compared to the 0% banana-mash treatment, with 1.4% viability observed after 6 day compared to 53% viability, respectively (**Figure 6.3**). The observed inactivation for the 0% banana-mash treatment was unexpected because the undissociated carboxylic acid concentrations were very low (**Figure 6.2C, A4.5B, A4.6B**). However, similar inactivation rates have been observed previously for treatments with low undissociated carboxylic acid concentrations, and they were attributed to additional fermentation products that were not measured (Harroff et al., 2017). One fermentation product that we thought might be important, particularly with added banana waste, was lactic acid. However, during a preliminary experiment we exposed *Ascaris* eggs to concentrations of lactic acid up to 1 M (at pH 2, for 1-15 days at 30°C) and we observed less than a 10% decrease in viability for all treatments (data not shown). Riungu et al. (2018) also tested *Ascaris* inactivation related to fermentation of UDDT-SW and food waste. The results are difficult to compare because individual carboxylic acid concentrations were not measured in the Riungu et al. study; however, inactivation below detection limits was observed within 4 days for many treatments. These inactivation rates were even faster than in our study, which can be partly attributed to the higher temperature of 35°C for the Riungu et al. study. Indeed, temperature has

been shown to have a significant impact on *Ascaris* inactivation rates by ammonia (Fidjeland et al., 2015). We anticipate a similar effect for carboxylic acids because both methods rely on the ability of the chemicals to cross the lipid membrane of the eggs (Fidjeland et al., 2015).

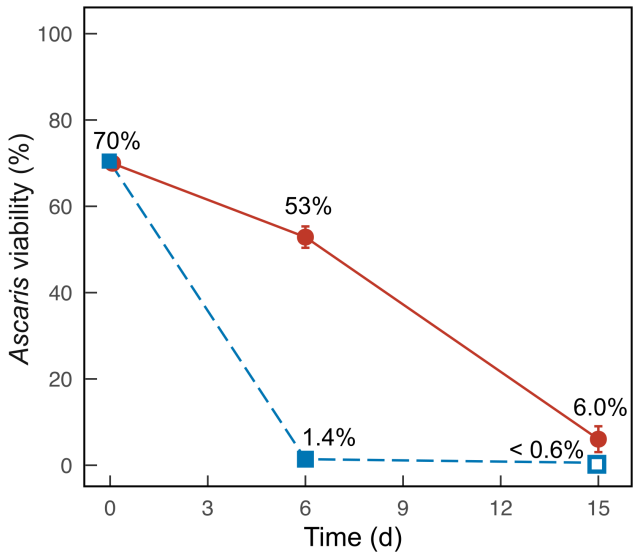

Figure 6.3 Average percent viability of *Ascaris* eggs collected from duplicate batch fermentation media bottles with banana mash added at 0% (m/m) (●) and 20% (m/m) (■). Error bars indicate the range between the two samples. Open symbols indicate that viability was below the detection limit (0.6 %).

6.5.3 Experiment 3: Field-scale trial

Warm temperatures are needed to produce carboxylic acids

We used data loggers to measure and record the temperature every 15 min in each of the 6 barrels that were used for the field-scale trial. Two of the loggers failed during the experiment, and the data was not retrievable. However, we can assume that the temperatures in all barrels were the same because the temperatures measured from the remaining four loggers were not statistically different (p = 0.235) (**Figure A4.7**). During the first 29 days of the trial, the barrels were kept outside under a black tarp. The average

temperature during this period was 22.1°C with a range of 17.7°C-29.0°C and standard deviation of 1.8°C. The observed temperatures were lower than we had originally anticipated, which resulted in very little carboxylic acid production during this period (**Table 6.3, Figure 6.4 and A4.8**). Previous fermentation trials that produced carboxylic acids from HFM or UDDT-SW, including the lab-scale trials here, were performed at higher temperatures of 30°C (Harroff et al., 2017) and 35°C (Riungu et al., 2018). Therefore, we moved the barrels to a greenhouse on Day 29 of the operating period. From Day 29 until the end of the trial on Day 56, the average temperature was 26.9°C with a range of 18.7°C-34.5°C and standard deviation of 2.2°C. This temperature increase resulted in modest increases in *n*-caproic acid concentrations between days 29 and 42 (**Figure 6.4A and 6.4B**), but concentrations remained lower than seen previously, likely because the average temperature remained below 30°C.

Table 6.3 Carboxylic acid concentrations at transitional time points during the field-scale trial for Experiment 3. Barrels were moved into a greenhouse on Day 29; banana waste was added to Treatments 1 and 2 on Day 42; and Day 56 was the final day of the trial. Banana waste was already added at Day 0 for Treatment 3.

Day	Treatment 1			Treatment 2			Treatment 3		
	***n*-Butyric acid (mM)**	***n*-Valeric acid (mM)**	***n*-Caproic acid (mM)**	***n*-Butyric acid (mM)**	***n*-Valeric acid (mM)**	***n*-Caproic acid (mM)**	***n*-Butyric acid (mM)**	***n*-Valeric acid (mM)**	***n*-Caproic acid (mM)**
0	55.8 (±5.7)	5.6 (±0.3)	2.0 (±0.04)	70.4 (±3.3)	7.1 (±2.0)	3.5 (±0.7)	41.2 (±0.1)	4.6 (±0.3)	2.5 (±0.2)
29	69.8 (±1.0)	15.7 (±2.4)	7.7 (±1.4)	73.3 (±2.0)	15.5 (±0.7)	4.0 (±0.5)	43.1 (±1.8)	5.7 (±1.2)	1.4 (±0.3)
42	68.8 (±2.8)	19.2 (±0.5)	12.6 (±1.1)	75.1 (±1.3)	16.0 (±0.8)	18.5 (±2.0)	58.0 (±13.4)	18.1 (±12.2)	24.7 (±21.1)
56	85.0 (±0.5)	22.2 (±4)	35.4 (±11.7)	84.0 (±6.4)	27.4 (±1.3)	43.1 (±5.5)	63.6 (±2.7)	25.4 (±2.7)	53.7 (±1.6)

If pH > 5 is maintained, then banana waste is rapidly converted to carboxylic acids, and high undissociated concentrations can be achieved without experiencing product inhibition

We added banana waste to Treatments 1 and 2 on Day 42 of the operating period as 20% of the total mass, which was after the temperature had been increased. As anticipated, the pH decreased within 2 days from 6.9 to 5.1 for Treatment 1 and from 6.3 to 5.2 for Treatment 2 (**Figure 6.4C**). However, the pH never decreased below 5.0 for either treatment. Based on the 20% banana-mash treatment during Experiment 2, we had anticipated a lower pH. We are attributing the higher pH for Experiment 3 to two possible reasons: (1) the different composition between banana mash without peel (Experiment 2) and banana waste with peel (Experiment 3); and (2) ammonia production from protein breakdown during the longer fermentation period compared to Experiment 2 (ammonia data not available), which would have caused a higher buffering capacity of the UDDT-SW (Procházka et al., 2012; Rose et al., 2015). For Treatment 3, we added banana waste from the beginning of the operating period (Day 0), and the pH was reduced to 4.5 within the first 3 days of the trial (**Figure 6.4D**), even though the temperature had been relative low. The pH consistently remained below the carboxylic acid pKa of 4.8 until Day 29 of the operating period (**Figure 6.4D**). However, after moving the barrels to the greenhouse on Day 29, the pH for Treatment 3 increased slightly to above 5.0 (**Figure 6.4D**), likely due to increased ammonia production coinciding with increased microbial activity. However, ammonia concentration data is not available to confirm this theory. From Day 32 until the end of the trial, the average pH further increased, but remained below 6 (**Figure 6.4D**).

We achieved average final *n*-caproic acid concentrations for Treatments 1, 2, and 3 of 35.4 mM, 43.1 mM, and 53.7 mM, respectively (**Table 6.3**). These concentrations were higher than we observed in any previous study or experiment. If we had not stopped the

trial on Day 56 of the operating period, the longer fermentation period would likely have resulted in even higher concentrations because the concentrations were still increasing. During Experiments 1 and 2, we observed a maximum average *n*-caproic acid concentration of 22.4 mM (**Figure 6.1B and 6.2B**), while the maximum average *n*-caproic acid concentration previously had been 27.1 mM (Harroff et al., 2017). The likely reason for the higher concentrations of *n*-caproic acid during Experiment 3, is the relatively high conversion efficiency of *n*-butyric acid into *n*-caproic acid, explaining the lower concentrations of *n*-butyric acid during Experiment 3 than what we have observed previously (**Table 6.3, Figure A4.8**) (Harroff et al., 2017).

After increasing the temperature, we observed a larger difference in results between the two barrels per treatment than before for both the carboxylic acid concentrations and the pH (**Figure 6.4 and A4.8**). Particularly for Treatment 3, one barrel consistently lagged behind the other one. Regardless of the precise onset of carboxylic acid production, banana waste was rapidly converted to carboxylic acids when the pH was above 5.0 at the higher temperature (**Figure 6.4A, B and A4.8A, B**).

Although the average pH in all treatments was above the pKa by the end of this experiment, a pH of 5 combined with the relatively high total concentrations of *n*-caproic acid resulted in higher concentrations of undissociated *n*-caproic acid concentrations than we have observed previously (**Figure 6.4C, D**). Final concentrations of undissociated *n*-caproic acid were 10.2 mM, 13.1 mM, and 12.6 mM for Treatments 1, 2, and 3, respectively (**Figure 6.4C, D**). The highest undissociated *n*-caproic acid concentration observed thus far was 7.1 mM in the 20% banana-mash treatment from Experiment 2. For Treatment 3, an unexpected trend for undissociated *n*-caproic acid was observed around

Day 44 of the operating period because the total concentration of *n*-caproic acid steadily increased as anticipated, but the undissociated concentration increased between Day 42 and 44 and then decreased between Day 44 and 46 (**Figure 6.4D**). This inconsistency occurred because of non-homogenous conditions within the barrels, resulting in changes in the average pH between sampling days. The pH on Day 44 was slightly lower than on Day 46 (**Figure 6.4D**), and small differences in pH result in large differences in undissociated carboxylic acid concentrations due to the exponential relationship between the two. This result is noteworthy because it demonstrates that non-homogenous conditions within a bioreactor can result in large differences in efficacy for pathogen inactivation.

Additional work is needed to optimize co-fermentation conditions

The results here suggest that banana waste (or alternative food waste) co-fermentation rates should be optimized to result in a pH of about 5, which will maximize undissociated carboxylic acid concentrations by promoting a large fraction of the total concentration to be in the undissociated form, while avoiding inhibition that appears to result at pH values below 4.8. One goal of the current study was to determine whether a two-stage (Treatments 1 and 2) or one-stage (Treatment 3) process was preferred for accomplishing this goal. The final concentrations of undissociated *n*-caproic acid were similar between the three treatments, but they were also still increasing when the experiment ended on day 56 (**Figure 6.4C and 6.4D**). With additional fermentation time, more differences may have been observed between the treatments. Therefore, it is difficult for us to make a firm conclusion about which treatment performed better. If the experiment is repeated

with temperatures at 30°C during the entire experiment, fermentation should occur faster, and differences between the treatments may become apparent with less time required. When resulting performances in the one-stage and two-stage processes are determined to be equivalent, then a one-stage process is strongly preferred because it is simpler to operate and implement. Additional work should also be performed to determine optimal ratios of food waste to UDDT-SW that will result in a pH of approximately 5.

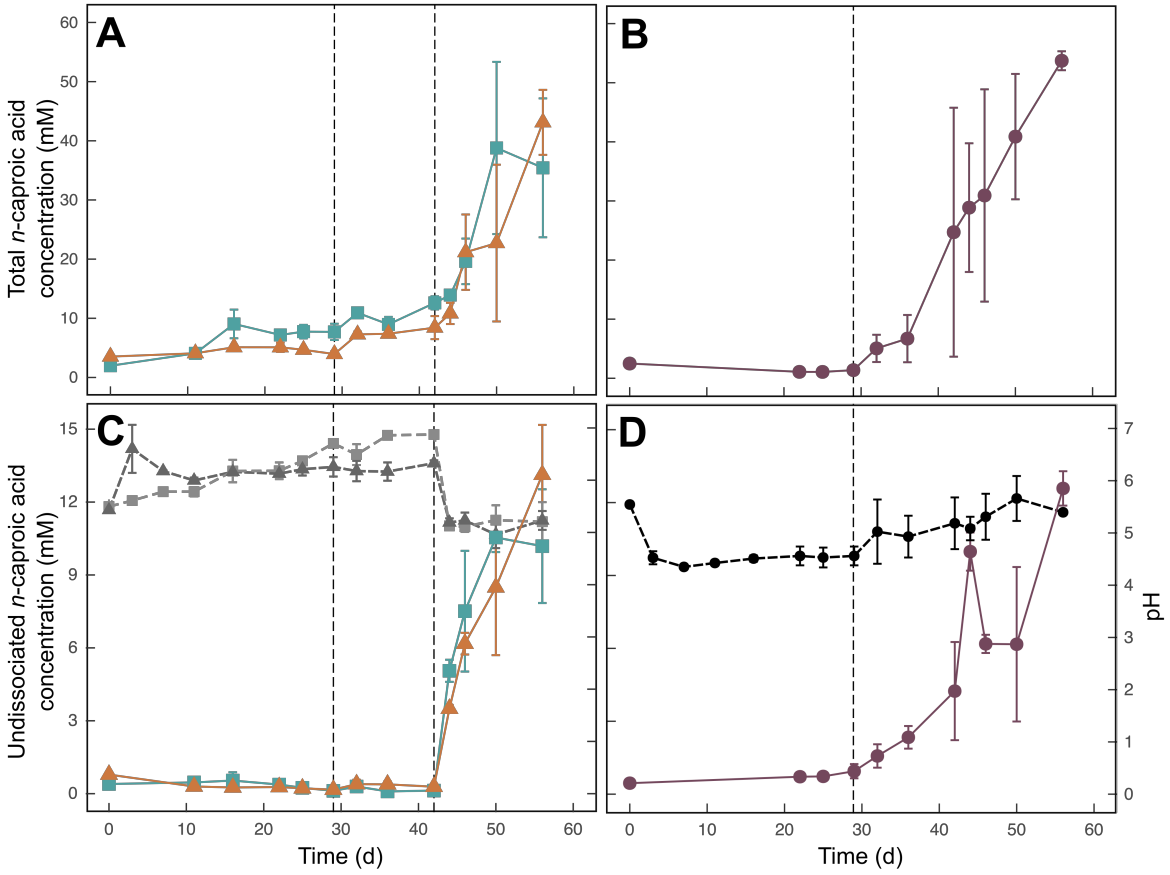

Figure 6.4 Total *n*-caproic acid concentrations (A and B), undissociated *n*-caproic acid concentrations, (C and D) and pH (C and D) for field-scale trial during Experiment 3. For C and D, undissociated *n*-caproic acid concentrations (solid colored lines) are plotted against the left axis, and pH (dashed, gray-scale lines) is plotted against the right axis. Treatments 1 (■) and 2 (▲), which were conducted in two stages are shown on the left (A and C). Treatment 3 (●), which had banana waste mixed with UDDT-SW at initial startup is shown on the right (B and D). Error bars show the range

between duplicate barrels. The dashed vertical line at Day 29 indicates the time that bioreactors were moved into the greenhouse, and the average temperature increased from 22.1°C to 26.9°C. The dashed vertical line at Day 42 in A and C indicates the time that banana waste was added to Treatments 1 and 2.

Ascaris inactivation in field-scale trial

We only collected *Ascaris* data at two time points (Days 11 and 29) during the field-scale trial and only during the low temperature period (**Table 6.4**). We had not anticipated the low temperatures and resulting slow fermentation rates during the first 29 days of the operating period. Even though we were able to continue the fermentation trial of Experiment 3 until Day 56, we were only able to monitor *Ascaris* inactivation until Day 29 due to the three-week incubation period required to determine *Ascaris* viability after the samples are collected. Therefore, our *Ascaris* data is very limited for the field trial. In summary, viabilities for the eggs exposed in the barrels were similar to the eggs contained in tubes of autoclaved UDDT-SW. The barrels simply did not have sufficiently high carboxylic acid concentrations during the first 29 days to cause any meaningful additional inactivation of the *Ascaris* eggs. Meanwhile, the inactivation of eggs that did occur in both the barrels and autoclaved UDDT-SW was due to an unmeasured compound or compounds that were present in the UDDT-SW prior to fermentation. Thus, further studies, which we outlined above, are still necessary to show *Ascaris* inactivation at the larger field scale.

Table 6.4 Average percent viability of *Ascaris* eggs collected for field-scale trial during Experiment 3. Viability is expressed as number of viable eggs/total number counted × 100. Standard deviation of four samples (two *Ascaris* chambers in each of two replicate barrels) is given in parentheses. The baseline viability of *Ascaris* eggs used in this experiment was 69.9%.

Time (d)	Percent viability (standard deviation)			
	Treatment 1	Treatment 2	Treatment 3	Autoclaved UDDT-SW
11	50.9 (17.9)	43.9 (9.1)	67.3 (3.1)	66.4 (5.0)
29	6.5 (4.9)	26.9 (12.0)	19.5 (8.8)	22.1 (8.9)

6.6 Conclusions

Several findings were made in this study that improve our understanding of how carboxylic acid fermentation can be used as part of a sanitation waste treatment system to inactive pathogens:

1. Fermentation of UDDT-SW in combination with food waste can be used to inactivate *Ascaris* eggs in a real sanitation setting when the carboxylic acid product spectrum is shifted toward longer-chain carboxylic acids, including *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid, rather than just acetic acid.
2. A specialized inoculum of carboxylic-acid-producing bacteria is not necessary because the gut microbiome found in UDDT-SW is already capable of producing carboxylic acids with longer carbon chains.
3. Fermentation temperatures should be higher than 30°C to promote reasonable rates of carboxylic acid production. Lower temperatures will result in longer required fermentation times or no carboxylic acid production at all. For waste treatment systems, long fermentation times require larger and more expensive bioreactors.

4. When easily degraded food waste, such as banana waste, is co-fermented with UDDT-SW in a way that maintains a pH between 4.8 and 5.2, we can accomplish our two main goals of: (1) producing high total concentrations of carboxylic acids; and (2) reducing the pH to cause a greater fraction of the total concentration to exist in the undissociated form. If the pH is below 4.8, then carboxylic acid production is inhibited. If the pH is above ~5.2, the fraction of carboxylic acids in the undissociated form is too low for the process to effectively inactivate pathogens.
5. The timing of food waste addition and ratio of food waste to UDDT-SW can both be optimized in a two-stage process to maintain an appropriate pH. However, for practical implementation of waste treatment systems, it is simpler to mix food waste and UDDT-SW when the fermentation is started. Therefore, an appropriate ratio of food waste should be found that could maintain the appropriate pH. This ratio will need to be adapted to individual systems based on composition and buffering capacity of the UDDT-SW and food waste used.
6. *Ascaris* eggs were inactivated faster when food waste was used to increase concentrations of undissociated carboxylic acids, but more investigation will be needed to determine appropriate operating conditions to inactivate pathogens, including *Ascaris* eggs.

6.7 Acknowledgements

We thank Daniel Kyalo, Davis Ireri, Clifford Odhiambo, Eunice Muthui, Ricky Ojwang, and Jimmy Kirui at Sanergy for valuable assistance in organizing logistics, assisting in

setup, and sampling for the field-scale trial. We also thank Lucinda Li for her work investigating *Ascaris* inactivation by lactic acid. LH acknowledges support from the Cross-Scale Biogeochemistry and Climate NSF IGERT program (Award #1069193) and the NSF Graduate Research Fellowship Program (DGE-1650441). LA acknowledges support from the Alexander von Humboldt Foundation in the framework of the Alexander von Humboldt Professorship endowed by the Federal Ministry of Education and Research in Germany.

6.7 Supporting information

Supporting information is provided in Appendix 4. It contains eight figures showing photographs from experimental setups, *n*-butyric acid and *n*-valeric acid data, and temperature measurements from the field-scale trial.

CHAPTER 7

SUMMARY AND RECOMMENDATIONS FOR FUTURE WORK

7.1 Summary

The results presented in this dissertation demonstrate that production and chain elongation of carboxylic acids within human fecal material (HFM) is a promising strategy for inactivating pathogens in sanitation waste treatment systems. In Chapter 3, I demonstrated initial feasibility and provided a deeper understanding of the influence of pH on *Ascaris* inactivation rates. I also developed initial relationships between *Ascaris* viability and concentration of *n*-butyric acid and *n*-caproic acid at four different exposure times. This was an important first step in determining time and concentration combinations that would result in adequate *Ascaris* inactivation. In Chapters 4 and 5, I expanded on these findings to incorporate effects of temperature and exposure to mixtures of multiple carboxylic acids. In Chapter 4, I specifically looked at the effect of temperature and found that *Ascaris* eggs can be inactivated at temperatures as low as 34°C, which contradicts current guidelines. I developed a more appropriate time-temperature relationship for *Ascaris* inactivation at mesophilic temperatures and demonstrated that it is still conservative, particularly if *Ascaris* eggs are exposed under aerobic conditions or in matrices of HFM or manure. In Chapter 5, I developed a logistic regression model to predict *Ascaris* inactivation as a function of five factors: (1) concentration of *n*-butyric acid, (2) concentration of *n*-valeric acid, (3) concentration of *n*-caproic acid, (4) exposure time, and (5) temperature. This model is useful for providing preliminary estimations of required operating conditions for real sanitation waste

treatment systems; however, it may over-predict inactivation rates in some cases. Therefore, *Ascaris* inactivation should be verified in individual treatment systems through testing. Finally, I performed a field-scale trial and demonstrated that real sanitation waste collected in Nairobi, Kenya can be fermented to produce carboxylic acids. Furthermore, co-fermentation of the sanitation waste with banana waste resulted in higher concentrations of *n*-caproic acid than had been observed in any previous trials. The banana waste also reduced the pH in fermentation systems to about 5, which increased the concentration of undissociated carboxylic acids and is critical for pathogen inactivation.

7.2 Future work: exploring matrix effects on Ascaris *inactivation*

The promising results found in this dissertation open many avenues for further investigation. First, several questions remain about the influence of matrix effects on the inactivation of *Ascaris* eggs. Most of the *Ascaris* work performed here was conducted by exposing *Ascaris* eggs to various conditions of time, temperature, and carboxylic acid concentrations within aqueous solutions. However, in real sanitation waste treatment systems, *Ascaris* eggs will be found within a matrix of HFM. In Chapter 3, I found that *Ascaris* eggs were inactivated faster in HFM that had not been spiked with carboxylic acids than in HFM that had been spiked with carboxylic acids, while *Ascaris* eggs contained in HFM that had been autoclaved showed very little inactivation. These results indicated that additional compounds were produced during fermentation that caused inactivation. The compounds were not carboxylic acids that I could measure, but I found one unidentified peak with my gas chromatography results. The peak eluted between the

standards for *n*-caprylic acid (C8) and *n*-pelargonic acid (C9). In future work, mass spectrometry should be used to identify this compound, and experiments with *Ascaris* eggs should be performed to confirm that it causes inactivation.

In Chapter 4, I found further evidence that additional compounds in HFM or manure matrices contribute to faster inactivation rates. The time-temperature relationship that I developed using data from exposure in aqueous solutions was consistently conservative compared to findings from literature that were performed within HFM or manure. In Chapter 5, this finding was contradicted because the model for predicting *Ascaris* inactivation was developed using data from aqueous exposures. Based on the results in Chapters 3 and 4, I expected the model to be conservative for predicting *Ascaris* inactivation in HFM matrices; however, I found that the model predicted faster inactivation rates than were observed in the Chapter 3 experiment with *Ascaris* eggs exposed in HFM.

The contradiction in results can be attributed to several factors. First, HFM is a complex matrix containing many compounds (e.g., ammonia, aldehydes, and ketones) that may have small effects on *Ascaris* viability. There are also many inconsistences within HFM matrices that may result in slower inactivation rates than expected. Pockets of cooler temperatures or lower concentrations of inactivating compounds may cause heterogeneity in inactivation results. Future work should deepen our understanding of the impact that these inconsistencies have on inactivation results and identify specific parameters that should be closely monitored in order to accurately predict *Ascaris* inactivation rates. This work should also provide better evidence about the transferability

between results found in aqueous exposures and those found in complex matrices or between results found in different complex matrices.

7.3 Future work: exploring effects of aerobic conditions on Ascaris *inactivation rates*

In Chapter 4, I found that mesophilic temperatures inactivated *Ascaris* eggs faster under aerobic conditions than under anaerobic conditions, but many questions remain unanswered about this effect. First, I only demonstrated the effect of aerobic conditions on thermal inactivation. Similiar experiments could be performed to confirm that the effect is also true for chemical inactivation of *Ascaris* eggs. I also only tested inactivation under strictly aerobic and strictly anaerobic conditions. Investigation of the effect of short-term or periodic exposure to aerobic conditions or the effect of micro-aerobic versus fully aerobic conditions would also be useful because those conditions are more representative of environmental conditions that *Ascaris* eggs will experience.

7.4 Future work: exploring important factors for scale-up and implementation

While the recommendations above provide important steps to improve our fundamental understanding of the parameters affecting *Ascaris* inactivation, the most important future work relates to implementing carboxylic acid production and chain elongation in real sanitation waste treatment systems. First, a new field-scale trial should be performed with the bioreactors kept at ~30°C from the initial setup. This is important for determining expected retention times that will be needed for fermentation. The rate of carboxylic acid production observed in my field-scale trial was slow and would require impractically

large bioreactors in full-scale systems. Warmer temperatures should improve production rates and reduce required bioreactor volumes.

Second, additional experimentation should be performed to determine appropriate co-fermentation conditions with food waste that will result in a pH near 5 and high concentrations of undissociated carboxylic acids. Parameters that can be altered include the type of food waste used and mixture ratios. Optimization of these parameters will likely vary considerably between different treatment systems, but preliminary work about this optimization will be useful.

Finally, the impact of pre-treatment fermentation on downstream processes should be evaluated. After carboxylic acid production and chain elongation, the initial stages of biological degradation have already occurred. The effluent from this process should then be converted to value-added products such as biogas and black soldier fly larvae more easily than raw sanitation waste. However, high concentrations of carboxylic acids may also have an inhibitory effect on downstream processes. In that case, an additional step may be needed to increase the pH in the fermentation effluent, thereby decreasing the concentrations of undissociated carboxylic acids, or to dilute the fermentation effluent with an additional organic waste stream.

7.5 Conclusions and implications

The bioprocess developed here has great potential for improving sanitation waste treatment methods around the globe, and it has several advantages over alternative processes. First, it is an anaerobic process, which is simpler to maintain and easier to operate than an aerobic process because it does not require aeration. Second, it relies on

the community of microorganisms that are naturally found in sanitation waste. Therefore, no external inoculum is needed, and it is more robust than a process that relies on a single organism. Finally, the cost of the bioprocess is low because the only required inputs are waste materials (sanitation waste and food waste). The work presented in this dissertation demonstrated that carboxylic acid production and chain elongation within HFM is an effective and feasible means of inactivating pathogens, and the future work described above will help determine appropriate operating requirements for scale-up and implementation.

APPENDIX 1

SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Table A1.1 Treatments for Experiment 2.

pH	[HA][a] *n*-Butyric Acid (mM)	[HA][a] *n*-Caproic Acid (mM)	[HA+A^-][b] *n*-Butyric Acid (mM)	[HA+A^-][b] *n*-Caproic Acid (mM)
2	100	10.0	100	10.0
2	135	13.5	135	13.5
2	170	17.0	170	17.0
2	205	20.5	205	20.5
2	240	24.0	240	24.0
4	100	10.0	115	11.3
4	135	13.5	155	15.3
4	170	17.0	196	19.2
4	205	20.5	236	23.2
4	240	24.0	276	27.2
5	100	10.0	251	23.2
5	135	13.5	339	31.3
5	170	17.0	427	39.4
5	205	20.5	515	47.5
5	240	24.0	603	55.6

[a] [HA] represents the concentration of the uncharged species only. The same five concentrations of [HA] were used at each pH level.
[b] [HA + A^-] represents the total concentration of uncharged acid *plus* charged conjugate base.

Table A1.2 Treatments for Experiment 3.

Acid	Exposure Time (d)	Concentrations Tested (mM)
n-Butyric	2	250, 275, 300*, 325, 350, 370*, 400, 450
	6	50, 100, 150*, 200, 250, 300*, 350, 400
	12	10, 25, 50*, 100, 150, 200*, 250, 300
	20	5, 10, 25*, 50, 100, 150*, 200, 250
n-Caproic	2	16, 20, 22*, 24, 26, 30*, 32, 36
	6	4, 6, 8*, 12, 16, 22*, 26, 30
	12	1, 2, 4*, 6, 8, 12*, 16, 22
	20	1, 2, 4*, 6, 8, 12*, 16, 20

*Duplicates were tested at these concentrations.

Table A1.3 Treatments for Experiment 4 and measured initial conditions.

Treatment Number	pH	*n*-Butyric Acid (uncharged acid *plus* conjugate base)	*n*-Caproic Acid (uncharged acid *plus* conjugate base)	Autoclaved? (Y/N)
1	Low (pH=4.72)	Spiked (269 mM)	Spiked (22.5 mM)	N
2	Low (pH=5.03)	No amendment (27.5 mM)	No amendment (3.1 mM)	N
3	Raised pH after adding carboxylic acids to equal pH of Treatment 4 (pH=6.30)	Spiked (243 mM)	Spiked (30.1 mM)	N
4	No adjustment (pH=6.40)	No amendment (36.1 mM)	No amendment (4.2 mM)	N
5	No adjustment (pH=6.50)	No amendment (16.7 mM)	No amendment (1.7 mM)	Y

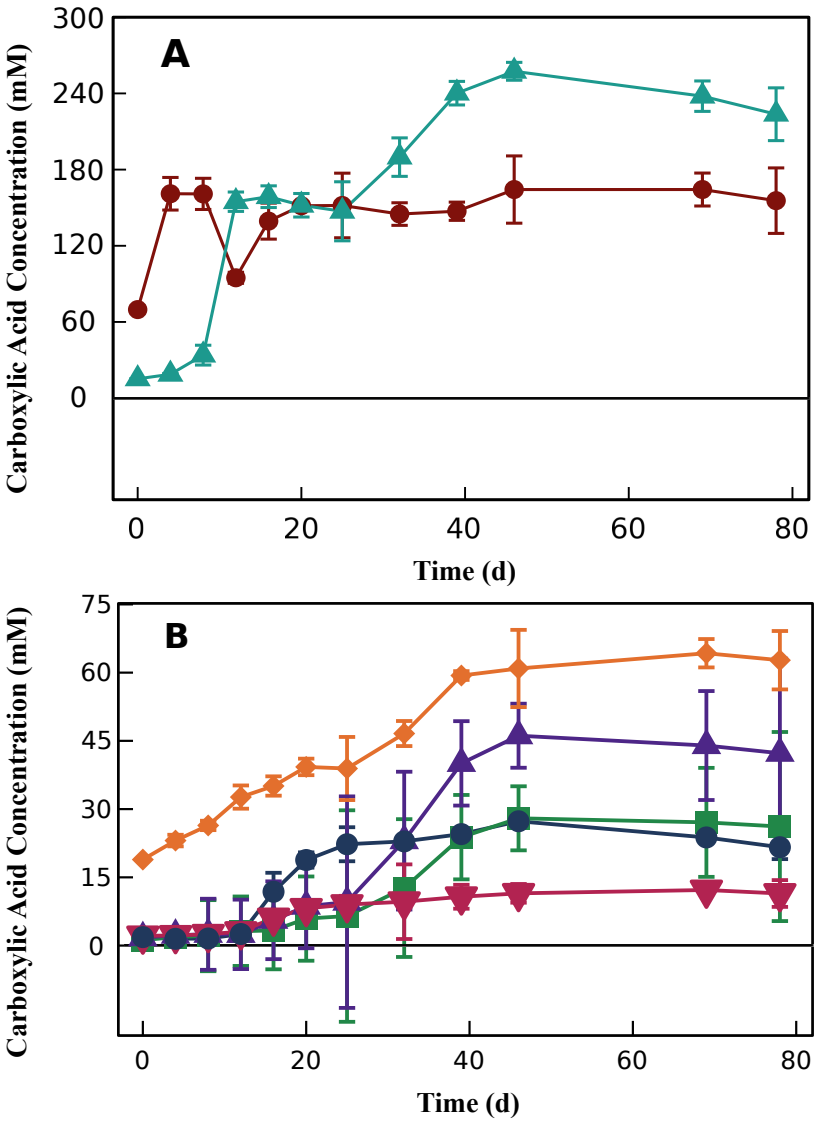

Figure A1.1 Accumulation of carboxylic acids from batch fermentation of HFM at 30°C. Panel A shows concentrations of acetic acid (●) and *n*-butyric acid (▲). Panel B shows concentrations of propionic acid (◆), *i*-butyric acid (■), *i*-valeric acid (▲), *n*-valeric acid (▼), and *n*-caproic acid (●). Concentrations represent the total concentrations of uncharged acid *plus* conjugate base. Error bars show the standard deviation of three biological replicates. Note that the scale of the y-axis is different in each panel.

Table A1.4 Individual model parameters and statistics at three different pH levels for inactivation of *Ascaris* eggs when exposed to *n*-butyric acid and *n*-caproic acid for 3 days at 37°C.

pH	Parameter *a* (SE[a])	*p*-value *a*	Parameter *b* (SE[a])	*p*-value *b*
2	0.0997 (1.40×10^{-2})	$1.41 \times 10^{-8*}$	146 (1.06)	$< 2 \times 10^{-16*}$
4	0.0936 (1.50×10^{-2})	$2.28 \times 10^{-7*}$	149 (0.944)	$< 2 \times 10^{-16*}$
5	0.106 (1.80×10^{-2})	$7.49 \times 10^{-7*}$	148 (0.775)	$< 2 \times 10^{-16*}$

The model presented in the main text incorporated the data from all three pH levels and was not statistically different from the model shown here with data separated by pH (p=0.0748).

[a] SE= standard error

[*] Significant at α=0.001

Table A1.5 Fraction of viable *A. suum* eggs for each treatment in Experiment 2 after exposure to carboxylic acids for 3 days at 37°C.

pH	Uncharged Carboxylic Acid Concentration[a] (mM) (SD*)	Total Carboxylic Acid Concentration[a] (Uncharged Acid *Plus* Conjugate Base) (mM) (SD[c])	Fraction Viable[b] (SD[c])
2	114 (1.41)	474 (6.70)	0.958 (0.046)
	151 (7.95)	630 (33.4)	0.376 (0.118)
	189 (8.06)	786 (34.2)	0.00377 (0.00144)
	230 (2.85)	958 (12.1)	0.00123[d] (0)
	267 (2.08)	1111 (8.49)	0.00132[d] (0)
4	110 (1.94)	526 (10.9)	0.955 (0.0312)
	152 (2.04)	722 (10.3)	0.453 (0.0368)
	188 (1.26)	916 (5.75)	0.00463 (0.00229)
	226 (1.22)	1108 (5.82)	0.00136[d] (0)
	268 (1.53)	1317 (7.99)	0.00210[d] (0)
5	106 (1.36)	1199 (15.5)	0.949 (0.0325)
	147 (1.77)	1636 (20.5)	0.532 (0.0364)
	180 (5.06)	2093 (59.3)	0.0143 (0.00783)
	209 (2.54)	2509 (30.1)	0.00151[d] (0)
	246 (2.07)	2946 (24.6)	0.00161[d] (0)
Controls			0.856 (0.013)

[a] Concentrations shown are combined concentrations of *n*-butyric acid and *n*-caproic acid. Each solution contained 10 mM *n*-butyric acid for every 1 mM *n*-caproic acid.
[b] Fraction viable is normalized to the average viability of the controls (*A. suum* eggs suspended in deionized water for 3 days at 37°C).
[c] SD= standard deviation of three replicates.
[d] Viability below detection limit.

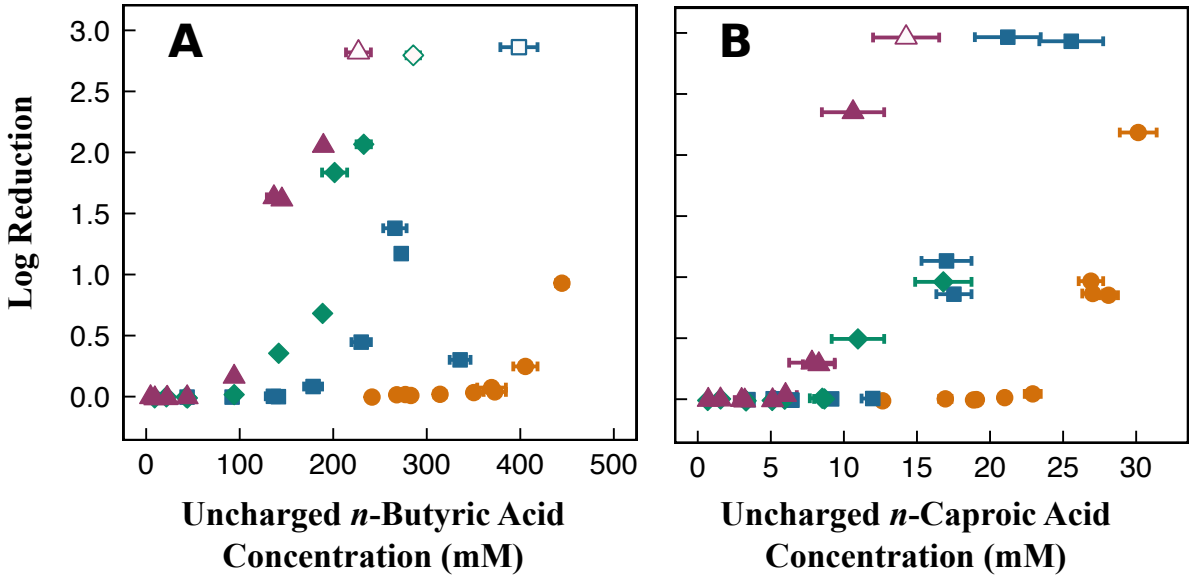

Figure A1.2 Log Reduction of *Ascaris* eggs due to exposure to *n*-butyric acid (A) and *n*-caproic acid (B). *Ascaris* eggs were exposed to carboxylic acids at 30°C for four different exposure times: 2 days (●), 6 days (■), 12 days (◆), and 20 days (▲). Open symbols indicate viability below the detection limit. The carboxylic acid concentration shown is the average of measurements made before and after the exposure period for each treatment, and the x-error bar shows the range between the two measurements. Replicate treatments are shown as individual points.

Table A1.6 Fraction of viable *A. suum* eggs for each treatment in Experiment 3.

***n*-Butyric Acid**			***n*-Caproic Acid**		
Exposure Time (d)	**Average Uncharged *n*-Butyric Acid Concentration[a] (mM)**	**Fraction Viable[b]**	**Exposure Time (d)**	**Average Uncharged *n*-Caproic Acid Concentration[a] (mM)**	**Fraction Viable[b]**
2	241	1.00	2	12.7	1.00
	268	0.966		16.9	0.993
	277	0.962		18.9	1.000
	283	0.979		19.1	1.000
	314	0.957		21.0	0.971
	350	0.927		22.9	0.905
	373	0.915		27.0	0.136
	369	0.843		26.9	0.108
	405	0.566		28.1	0.141
	444	0.118		30.1	0.007
	Controls	0.908 (0.0108[d])		Controls	0.908 (0.0108[d])
6	43.7	1.00	6	3.44	1.00
	91.9	1.00		5.16	0.991
	141	1.00		6.45	1.00
	136	0.994		6.37	1.00
	178	0.827		9.16	0.994
	230	0.358		12.0	0.987
	266	0.042		17.5	0.138
	273	0.067		17.0	0.074
	336	0.500		21.2	0.00108
	399	0.00126[c]		25.6	0.00117
	Controls	0.917 (0.0104[d])		Controls	0.917 (0.0104[d])
12	9.06	1.00	12	0.680	1.00
	21.6	1.00		1.56	0.995
	42.7	1.00		3.31	1.00
	44.0	1.00		3.28	1.00
	94.1	0.963		5.09	1.00
	142	0.442		5.95	0.999
	189	0.208		8.52	0.982
	201	0.015		8.67	0.995
	233	0.009		11.0	0.321
	286	0.00146[c]		16.8	0.109
	Controls	0.913 (0.0116[d])		Controls	0.913 (0.0116[d])
20	4.66	1.00	20	0.718	1.00
	9.26	1.00		1.56	1.00
	22.6	1.00		3.00	1.00
	22.2	1.00		3.21	1.00
	43.9	1.00		5.12	1.00
	94.1	0.688		6.01	0.925
	145	0.024		7.81	0.502
	137	0.023		8.28	0.518
	189	0.009		10.6	0.004
	227	0.00137[c]		14.3	0.00099[c]
	Controls	0.904 (0.0276[d])		Controls	0.904 (0.0276[d])

[a] Concentration is the average of measurements made before and after the exposure period.
[b] Fraction viable is normalized to the average viability of the controls for each exposure period.
[c] Viability below detection limit
[d] Standard deviation of three replicates

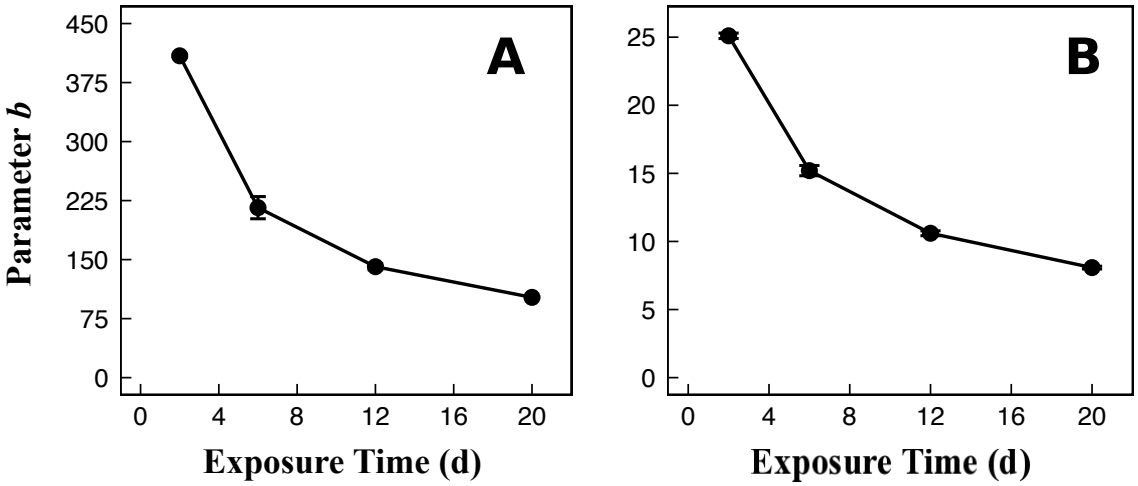

Figure A1.3 Model parameter *b* at the four different exposure times tested in Experiment 2 for *n*-butyric acid (A) and *n*-caproic acid (B). Error bars represent the standard error of the parameter *b*.

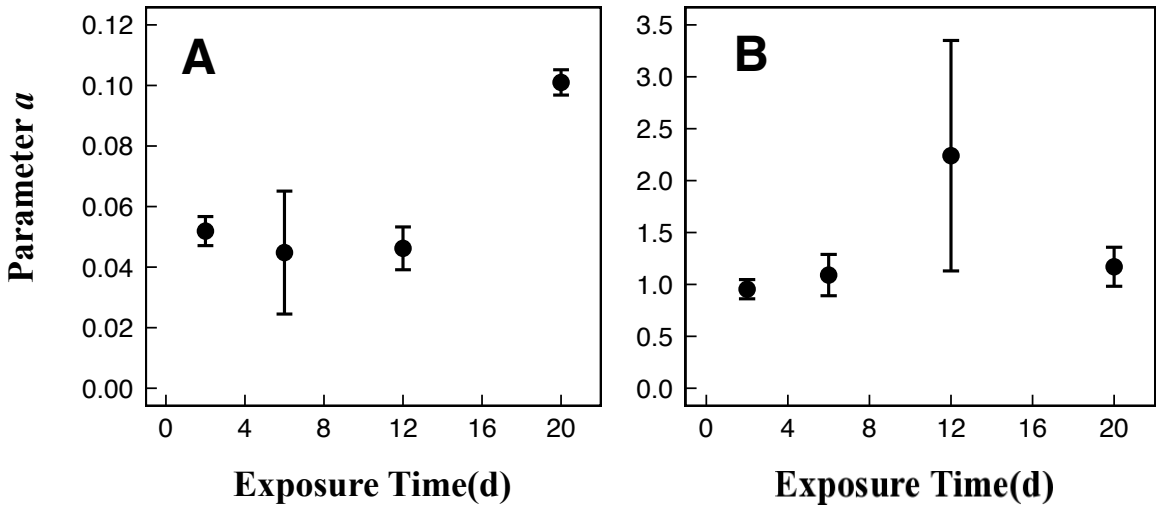

Figure A1.4 Model parameter *a* at the four different exposure times tested in Experiment 2 for *n*-butyric acid (A) and *n*-caproic acid (B). Error bars represent the standard error of the parameter *a.*

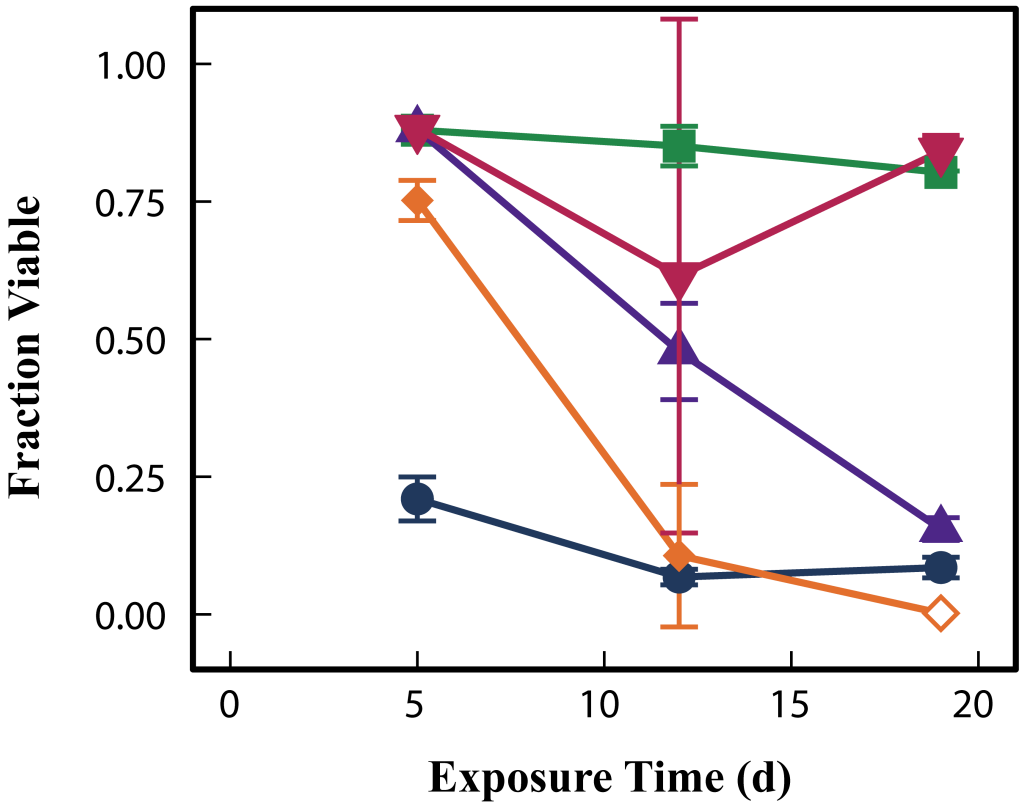

Figure A1.5 Inactivation of *Ascaris* eggs in HFM after exposure times of 5, 12, and 19 days. *A. suum* eggs were exposed to five treatments at 30°C : Treatment 1 with spiked acids and low pH (●); Treatment 2 with no added acids and low pH (■); Treatment 3 with spiked acids and natural pH (▲); Treatment 4 with no added acids and natural pH (◆); and Treatment 5 with autoclaved HFM, no added acids, and natural pH (▼). Error bars represent standard deviation of viabilities for three replicates. Open symbols indicate that viability was below the detection limit of 0.004.

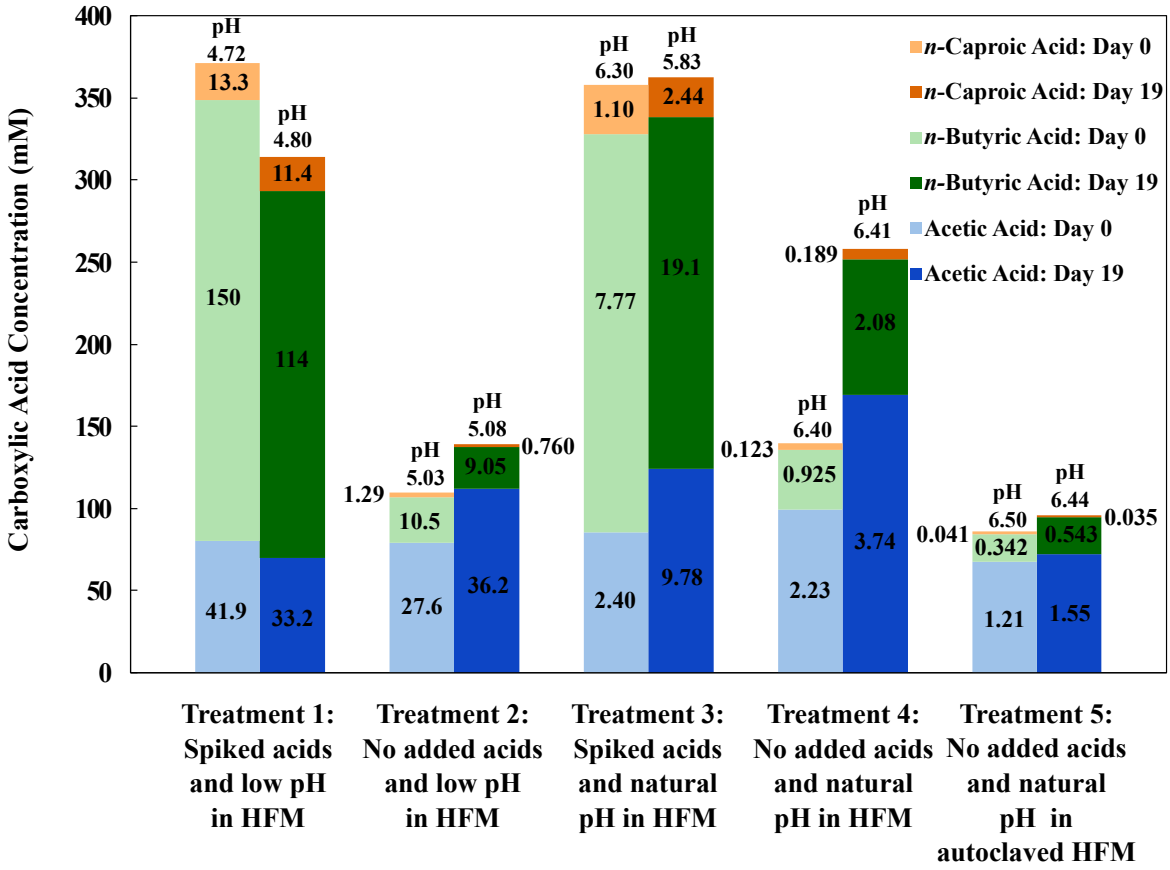

Figure A1.6 Concentrations of carboxylic acids measured at onset of experiment and after 19-day exposure period for Experiment 4. The height of each bar represents the total carboxylic acid concentration (uncharged acid *plus* conjugate base). The pH is shown on top of each bar, and the calculated uncharged carboxylic acid concentration is written within the bar.

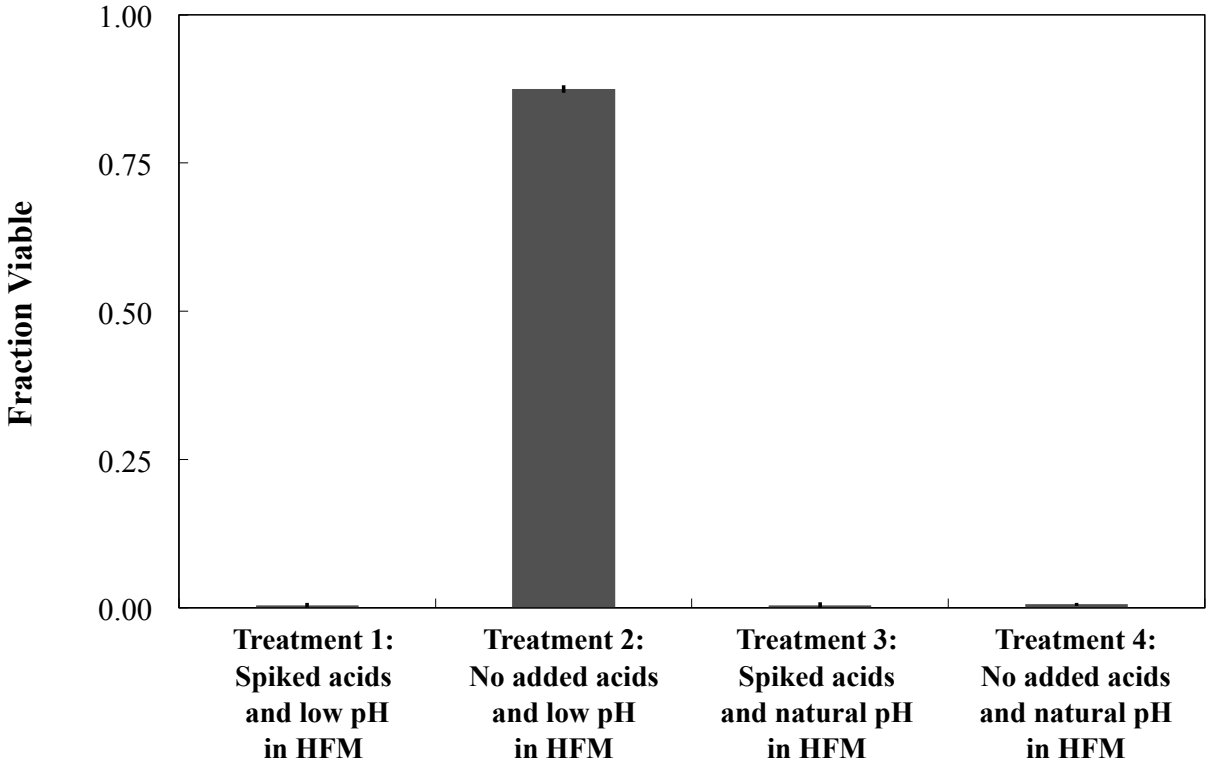

Figure A1.7 Previous experiment inactivating *A. suum* eggs in HFM. *A. suum* eggs were exposed to treatment conditions in HFM for 14 days at 30°C. For treatments with spiked acids, *n*-butyric acid and *n*-caproic acid were added to achieve final concentrations of 240 mM and 24 mM (uncharged acid plus conjugate base), respectively. For treatments with low pH, the pH was adjusted to 5.10 (+/- 0.05) using HCl. All other methods were the same as those for Experiment 4. Error bars represent standard deviation of viabilities for three replicates. Viabilities for Treatments 1, 3, and 4 were 0.004, 0.004, and 0.003, respectively. The detection limit was 0.004.

APPENDIX 2

SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Table A2. 1 Measured treatment conditions and experimental results.

Trial #	An/aerobic	Exposure Time (d)	Treatment temp. (°C)	Measured average temp. (°C)	Temp. standard deviation (°C)	Baseline percent viability (%)	Number of viable eggs	Number of not viable eggs	Percent viability* (%)	Log reduction**
1	Anaerobic	10.0	36	36.54	0.36	73.6	397	128	102.8	-0.01
1	Anaerobic	10.0	36	36.54	0.36	73.6	361	143	97.3	0.01
1	Anaerobic	15.1	36	36.67	0.41	73.6	137	370	36.7	0.44
1	Anaerobic	15.1	36	36.67	0.41	73.6	362	141	97.8	0.01
1	Anaerobic	20.1	36	36.77	0.41	73.6	353	174	91.0	0.04
1	Anaerobic	20.1	36	36.77	0.41	73.6	147	356	39.7	0.40
1	Anaerobic	23.9	36	36.44	0.34	73.6	327	236	78.9	0.10
1	Anaerobic	23.9	36	36.44	0.34	73.6	314	190	84.7	0.07
1	Anaerobic	30.1	36	36.56	0.25	73.6	273	236	72.9	0.14
1	Anaerobic	30.1	36	36.56	0.25	73.6	280	222	75.8	0.12
1	Anaerobic	42.0	36	36.45	0.44	73.6	182	320	49.3	0.31
1	Anaerobic	42.0	36	36.45	0.44	73.6	176	341	46.3	0.33
1	Aerobic	10.0	36	36.54	0.36	73.6	3	535	0.8	2.12
1	Aerobic	10.0	36	36.54	0.36	73.6	8	545	2.0	1.71
1	Aerobic	15.1	36	36.67	0.41	73.6	0	1023	0.1	2.88
1	Aerobic	15.1	36	36.67	0.41	73.6	0	1003	0.1	2.87
1	Aerobic	30.1	36	36.56	0.25	73.6	0	1042	0.1	2.88
1	Aerobic	30.1	36	36.56	0.25	73.6	0	1000	0.1	2.87
2	Anaerobic	4.00	40	40.06	0.28	67.5	200	300	59.3	0.227
2	Anaerobic	4.00	40	40.06	0.28	67.5	240	280	68.4	0.165
2	Anaerobic	8.00	40	39.88	0.30	67.5	105	411	30.2	0.521
2	Anaerobic	8.00	40	39.88	0.30	67.5	91	481	23.6	0.627
2	Anaerobic	11.9	40	39.63	0.50	67.5	12	535	3.3	1.49
2	Anaerobic	11.9	40	39.63	0.50	67.5	6	523	1.7	1.77
2	Anaerobic	16.0	40	39.78	0.51	67.5	2	347	0.8	2.07
2	Anaerobic	16.0	40	39.78	0.51	67.5	1	304	0.5	2.31
2	Aerobic	2.00	40	40.42	0.26	67.5	299	210	87.1	0.0601
2	Aerobic	2.00	40	40.42	0.26	67.5	285	231	81.9	0.0869
2	Aerobic	4.00	40	40.68	0.34	67.5	178	332	51.7	0.286
2	Aerobic	4.00	40	40.68	0.34	67.5	160	349	46.6	0.332
2	Aerobic	8.00	40	40.51	0.34	67.5	0	619	0.2	2.62
2	Aerobic	8.00	40	40.51	0.34	67.5	0	860	0.2	2.76
2	Aerobic	11.9	40	40.39	0.39	67.5	2	991	0.3	2.52
2	Aerobic	11.9	40	40.39	0.39	67.5	4	794	0.7	2.13

Table A2.1 (Cont.) Measured treatment conditions and experimental results.

Trial #	An/aerobic	Exposure Time (d)	Treatment temp. (°C)	Measured average temp. (°C)	Temp. standard deviation (°C)	Baseline percent viability (%)	Number of viable eggs	Number of not viable eggs	Percent viability* (%)	Log reduction**
2	Anaerobic	0.75	45	45.18	0.24	67.5	245	310	65.4	0.184
2	Anaerobic	0.75	45	45.18	0.24	67.5	225	277	66.4	0.178
2	Anaerobic	2.00	45	45.12	0.28	67.5	0	884	0.2	2.78
2	Anaerobic	2.00	45	45.12	0.28	67.5	0	742	0.2	2.70
2	Anaerobic	4.00	45	45.08	0.34	67.5	0	771	0.2	2.72
2	Anaerobic	4.00	45	45.08	0.34	67.5	1	994	0.1	2.83
2	Anaerobic	5.00	45	45.07	0.32	67.5	0	703	0.2	2.68
2	Anaerobic	5.00	45	45.07	0.32	67.5	0	976	0.2	2.82
2	Aerobic	0.75	45	45.22	0.06	67.5	85	419	25.0	0.602
2	Aerobic	0.75	45	45.22	0.06	67.5	100	420	28.5	0.545
2	Aerobic	1.00	45	45.11	0.36	67.5	14	489	4.1	1.38
2	Aerobic	1.00	45	45.11	0.36	67.5	44	461	12.9	0.889
2	Aerobic	2.00	45	45.27	0.35	67.5	0	808	0.2	2.74
2	Aerobic	2.00	45	45.27	0.35	67.5	0	893	0.2	2.78
2	Aerobic	4.00	45	45.28	0.44	67.5	0	813	0.2	2.74
2	Aerobic	4.00	45	45.28	0.44	67.5	0	741	0.2	2.70
3	Aerobic	1.99	36	36.54	0.28	67.5	357	239	88.8	0.0517
3	Aerobic	1.99	36	36.54	0.28	67.5	336	202	92.6	0.0335
3	Aerobic	5.03	36	36.40	0.32	67.5	168	354	47.7	0.321
3	Aerobic	5.03	36	36.40	0.32	67.5	153	365	43.8	0.359
3	Aerobic	8.00	36	36.30	0.29	67.5	45	472	12.9	0.889
3	Aerobic	8.00	36	36.30	0.29	67.5	36	474	10.5	0.980
3	Aerobic	11.9	36	36.19	0.29	67.5	5	517	1.42	1.85
3	Aerobic	11.9	36	36.19	0.29	67.5	9	498	2.63	1.58
4	Aerobic	2.77	34	34.22	0.27	69.1	289	221	82.0	0.0862
4	Aerobic	2.77	34	34.22	0.27	69.1	284	241	78.3	0.106
4	Aerobic	4.69	34	34.19	0.23	69.1	211	337	55.7	0.254
4	Aerobic	4.69	34	34.19	0.23	69.1	217	371	53.4	0.272
4	Aerobic	10.0	34	34.25	0.19	69.1	38	508	10.1	1.00
4	Aerobic	10.0	34	34.25	0.19	69.1	25	490	7.02	1.15
4	Aerobic	14.6	34	34.26	0.15	69.1	0	1005	0.14	2.84
4	Aerobic	14.6	34	34.26	0.15	69.1	0	1023	0.14	2.85
4	Aerobic	19.8	34	34.27	0.14	69.1	0	1000	0.14	2.84
4	Aerobic	19.8	34	34.27	0.14	69.1	0	1000	0.14	2.84

Table A2.1 (Cont.) Measured treatment conditions and experimental results.

Trial #	An/aerobic	Exposure Time (d)	Treatment temp. (°C)	Measured average temp. (°C)	Temp. standard deviation (°C)	Baseline percent viability (%)	Number of viable eggs	Number of not viable eggs	Percent viability* (%)	Log reduction**
4	Anaerobic	5.96	37	36.94	0.09	69.1	412	230	92.9	0.0322
4	Anaerobic	5.96	37	36.94	0.09	69.1	387	219	92.4	0.0343
4	Anaerobic	11.3	37	36.98	0.09	69.1	316	210	86.9	0.0608
4	Anaerobic	11.3	37	36.98	0.09	69.1	289	237	79.5	0.100
4	Anaerobic	15.9	37	37.03	0.15	69.1	222	293	62.4	0.205
4	Anaerobic	15.9	37	37.03	0.15	69.1	254	284	68.3	0.165
4	Anaerobic	21.1	37	37.03	0.14	69.1	211	291	60.8	0.216
4	Anaerobic	21.1	37	37.03	0.14	69.1	167	382	44.0	0.356
4	Anaerobic	40.0	37	37.11	0.14	69.1	0	1000	0.14	2.84
4	Anaerobic	40.0	37	37.11	0.14	69.1	14	457	4.30	1.37
4	Anaerobic	5.19	39	39.00	0.04	69.1	364	256	85.0	0.0708
4	Anaerobic	5.19	39	39.00	0.04	69.1	301	215	84.4	0.0736
4	Anaerobic	11.3	39	39.03	0.03	69.1	191	378	48.6	0.314
4	Anaerobic	11.3	39	39.03	0.03	69.1	176	351	48.3	0.316
4	Anaerobic	15.9	39	39.14	0.13	69.1	62	477	16.6	0.779
4	Anaerobic	15.9	39	39.14	0.13	69.1	32	491	8.85	1.05
4	Anaerobic	21.1	39	39.20	0.23	69.1	4	498	1.15	1.94
4	Anaerobic	21.1	39	39.20	0.23	69.1	3	998	0.43	2.36
4****	Anaerobic	32.0	39	39.20	0.25	69.1	0	1025	0.14	2.85

* Percent viability calculated as: $\frac{\text{Number of viable eggs/Sum of eggs counted}}{\text{Baseline \% viability}} \times 100$.

If no viable eggs were counted, percent viability is reported as equal to the detection limit: $\frac{1/\text{Sum of eggs counted}}{\text{Baseline \% viability}} \times 100$.

** Log reduction calculated as: $\text{Log}_{10}\left(\frac{\text{Baseline \% viability}}{\text{Number of viable eggs/Sum of eggs counted}}\right)$.

**** Duplicate sample for anaerobic exposure at 39°C for 32 days dried out during incubation, and eggs were not counted.

Table A2.2 Details and context for literature values discussed in the text and used in Figure 4.2.

Reference	Matrix	Anaerobic/ aerobic	Temp-erature (°C)	Inactivation time (degree of inactivation)	Egg source and type	Notes
Cruz Espinoza et al., 2012	Human fecal material with lime and soil additive (1 part lime: 60 part soil) to represent composting toilet, pH 8.30, total solids 73.6%, 139.72 mg/L or 7.8 mM ammonia, reported as ammonia gas plus ammonia in fecal material	Anaerobic	35	Between 14 d and 21 d** (≥99%)	*A, suum* eggs (likely from pig feces based on purchase from Excelsior Sentinel Inc.)	Eggs in 20 µm nylon mesh bags submerged in individual sealed containers with 70 g fecal material. Duplicate samples collected after 1, 3, 7, 14, 21, 28, 35, and 42 days with 100-855 eggs counted per sample. Viabilities at points before indicated inactivation time not given.
			40	Between 3 d and 7 d** (≥99%)		
			45	< 1 d** (≥99%)		
Ghiglietti et al., 1995	Aqueous (pH 7 saline solution)	Aerobic	40	14 d* (99%)	*A. suum* eggs from uteri of female worms	1% viable after 14 d and below detection after 21 d.
			40	Between 7 d and 14 d** (99%)	*A. lumbricoides* eggs	Eggs tested from female worms and human feces. Eggs from worms were 10% viable after 7 d and below detection after 14 d. Eggs from feces were 1% viable after 7 d and below detection after 14 d.
Johansen et al., 2013	Aqueous (1 mL sterile water in microcentrifuge tubes)	Not specified, likely somewhat aerobic	37	Between 2 d and 10 d (~99.6%)	*A. suum* eggs from deposited pig feces	250 eggs used in each treatment; exact number counted not indicated. Viability determined by hatching, not embryonation. Baseline viability was only slightly over 40%. No samples collected between 2 d and 10 d. Viability was 20-30% after 2 d and below
	Cattle slurry, lab-scale anaerobic digester	Anaerobic	37	Between 2 d and 10 d** (~99.6%)		
Johnson, Dixon, and Ross, 1998	Mesophilic sewage sludge digester	Anaerobic	35	> 35 d (50% inactivation at 35 d)	*A. suum* eggs from pig feces	Eggs in 20 um nylon bags sunk at least 1 m into digester. Tested unembryonated and embryonated eggs. Unembryonated eggs were 50% viable after 5 weeks; embryonated eggs remained > 98% viable after 5 weeks.

Table A2.2 (Cont.) Details and context for literature values discussed in the text and used in Figure 4.2.

Reference	Matrix	Anaerobic/ aerobic	Temp-erature (°C)	Inactivation time (degree of inactivation)	Egg source and type	Notes
Kato, Fogarty, and Bowman, 2003	Aqueous (DI water)	Not indicated	37	> 10 d (~84% inactivation at 10 d)	*A. suum* eggs from pig feces	Aqueous treatments took place in 1 mL DI water in microcentrifuge tubes. For anaerobic digester treatments, some eggs were spiked directly into 1-L semi-continuous anaerobic digesters, and others were contained in plastic chambers sealed with 30 µm mesh. Sampling ended after 10 d. Aerobic digesters were also tested, but the internal temperature was uncertain due to the air that was circulated to keep them aerobic.
			47	< 2 d (99%)		
	Anaerobic digester, thickened primary biosolids	Anaerobic	37	> 10 d (75% inactivation at 10 d)		
			47	< 2 d (99%)		
Manser et al., 2015	Swine manure slurry anaerobic digester (280 g manure, 1500 mL water, and urea to maintain ~1000 mg/L NH_3-N)	Anaerobic	35	24 d* (99.7%)	*A. suum* eggs from pig feces	99.7% inactivation based on given minimum of 360 eggs counted per sample. Inactivation time based on first exposure time found with no viable eggs. Samples were collected every 2 d.
	Aqueous (0.01 M phosphate buffer with 0.027 M KCl, 0.137 M NaCl, 1.9 g/L urea, and 0.6 g/L sodium acetate to mimic concentration in digesters, pH 7.9)	Anaerobic	35	24 d* (99.7%)		
		Aerobic	35	16 d* (99.7)		
Nordin, Nyberg, and Vinnerås, 2009	Unamended human fecal material, 17% dry matter, pH 8.1-8.3, $NH_{3(aq)}$=43 mM	Anaerobic	34	21 d* (99%)	*A. suum* eggs from uteri of female worms	10,000 eggs in 35 µm mesh bags, which were inserted in 200g of fecal material in sealed containers, sealed in a 50 mL tube containing urine solution, or stored in 0.9% NaCl solution. For urine treatment, some oxygen likely introduced when bags were removed after 1, 2, 7, and 10 d. Inactivation time calculated based on linear regression of inactivation.
	Aqueous (Urine diluted 1 part urine to 3 parts tap water, pH 8.7, $NH_{3(aq)}$=40 mM)	Not indicated but likely aerobic due to sampling frequency	34	8.5 d* (99%)		
	0.9% NaCl solution	Not indicated, storage conditions not given	34	> 31 d (7% inactivation after 31 d)		
Pecson et al., 2007	Municipal sludge from primary treatment plant near Mexico City, 5% total solids, pH 7	Anaerobic	40	14 d* (99%)	Naturally occurring *A. lumbricoides* eggs in sewage sludge	Inactivation time calculated based on estimated inactivation model.

Table A2.2 (Cont.) Details and context for literature values discussed in the text and used in Figure 4.2.

Reference	Matrix	Anaerobic/ aerobic	Temp-erature (°C)	Inactivation time (degree of inactivation)	Egg source and type	Notes
Scheinemann et al., 2015	Autoclaved cow manure with *Lactobacillaceae* culture added, initial pH 6.70 decreased to 4.94 after 3 d.	Anaerobic	37*	Between 21 d and 56 d** (99.7%)	*A. suum* eggs	~81,000 eggs added to 27 g of matrix, sealed in 50 mL tubes and stored in anaerobic containers. Samples collected after 0, 7, 21, and 56 d. 300 eggs counted per sample, in triplicate. For all treatments, no inactivation was observed after 21 d, and no viable eggs were found after 56 d.
	Non-autoclaved cow manure with *Lactobacillaceae* culture added, initial pH 6.68 decreased to 5.37 after 3 d.	Anaerobic	37*	Between 21 d and 56 d** (99.7%)		
	Aqueous (tap water)	Not indicated, no storage information given	37	Between 21 and 56 d** (99.7%)		
Senecal et al., 2018	5 mL phosphate buffer and 0.1 g human fecal material	Not clearly indicated, but minor oxygen exposure is assumed because some larval development was observed in a similar treatment at 20 °C.	42	6.2 d (99.9%)	*A. suum* eggs from intestinal feces of slaughtered pigs	Eggs mixed with phosphate buffer and fecal material in 7 mL tubes that remained sealed throughout exposure period. Inactivation time predicted using nonlinear regression.
Tharaldsen and Helle, 1989	Pig manure slurry	Aerobic (mechanically aerated composting tank)	37	Between 14 d and 22 d** (99%)	*A. suum eggs* from uteri of female worms	Eggs contained in 20 µm mesh bags. Eggs were 4% viable after 14 d. No viable eggs found at 22 d. 1% viability found at 24 d and 28 d. No viable eggs found at 31-49 d.
Current	Aqueous (0.1 N H_2SO_4)	Anaerobic	36	465.4 d (99.9%)	*A. suum* eggs from intestinal feces of slaughtered pigs	Calculated using linear regression. Anaerobic 36 °C and 37 °C conditions are extrapolated. The longest exposure time tested at 36 °C was 42 d, and eggs were 35% viable. The longest exposure time tested at 37 °C was 40 d, and eggs were 2.2% viable.
			37	57.5 d* (99.9%)		
			39	31.8 d* (99.9%)		
			40	20.5 d* (99.9%)		
			45	2.12 d* (99.9%)		
	Aqueous (0.1 N H_2SO_4)	Aerobic	34	16.6 d* (99.9%)		
			36	16.5 d* (99.9%)		
			40	12.7 d* (99.9%)		
			45	2.13 d* (99.9%)		

* Inactivation time was used in Figure 4.2.
** When a range of days is given for inactivation time, the longer time was used in Figure 4.2.

APPENDIX 3

SUPPLEMENTARY INFORMATION FOR CHAPTER 5

Table A3.1 Summary of treatment conditions and results for Group 1.

Treat-ment #	Coded factors [a]	*n*-Butyric acid (mM) [b]	*n*-Valeric acid (mM) [b]	*n*-Caproic acid (mM) [b]	Exposure time (d)	Temp (°C) [b]	Viability 1 (%)[c]	Viability 2 (%)[c]
1	0, 0, 0, 0, 0	275.0 (± 2.3)	58.6 (± 1.6)	17.7 (± 1.6)	9.9	29.9 (0.6)	0.2	*nd*
2	0, 0, 0, 0, 0	267.6 (± 5.1)	57.9 (± 2.2)	17.4 (± 1.9)	9.9	29.9 (0.6)	0.2	*nd*
3	0, 0, 0, 0, 0	275.1 (± 2.4)	60.3 (± 0.2)	18.1 (± 1.2)	9.9	29.9 (0.6)	*bd*	*nd*
4	0, 0, 0, 0, 0	277.9 (± 5.2)	58.5 (± 1.6)	17.8 (± 1.6)	9.9	29.9 (0.6)	0.1	*nd*
5	0, 0, 0, 0, 0	271.8 (± 0.9)	59.6 (± 0.6)	17.9 (± 1.5)	9.9	29.9 (0.6)	*bd*	*nd*
6	0, 0, 0, 0, 0	260.0 (± 12.6)	58.4 (± 1.8)	16.3 (± 3.0)	9.9	29.9 (0.6)	0.4	*nd*
7	-1, -1, -1, -1, -1	157.2 (± 1.7)	33.0 (± 1.1)	10.2 (± 0.5)	5.8	23.8 (1.0)	63.8	*nd*
8	1, -1, -1, -1, -1	381.0 (± 0.1)	35.5 (± 1.6)	9.8 (± 0.6)	5.8	23.8 (1.0)	0.4	*nd*
9	-1, 1, -1, -1, -1	138.5 (± 4.1)	83.6 (± 4.8)	10.5 (± 0.7)	5.8	23.8 (1.0)	7.8	*nd*
10	1, 1, -1, -1, -1	441.4 (± 12.0)	84.2 (± 2.0)	10.3 (± 0.5)	5.8	23.8 (1.0)	*bd*	*nd*
11	-1, -1, 1, -1, -1	159.3 (± 5.8)	32.5 (± 0.3)	26.1 (± 0.9)	5.8	23.8 (1.0)	2.8	*nd*
12	1, -1, 1, -1, -1	388.2 (± 2.4)	34.3 (± 0.3)	26.6 (± 0.5)	5.8	23.8 (1.0)	0.1	*nd*
13	-1, 1, 1, -1, -1	158.9 (± 3.3)	81.1 (± 0.6)	25.6 (± 0.7)	5.8	23.8 (1.0)	0.1	*nd*
14	1, 1, 1, -1, -1	426.5 (± 23.4)	85.6 (± 2.6)	26.8 (± 1.5)	5.8	23.8 (1.0)	*bd*	*nd*
15	-1, -1, -1, 1, -1	145.5 (± 10.0)	31.4 (± 2.7)	9.1 (± 1.7)	13.9	23.8 (1.0)	0.7	*nd*
16	1, -1, -1, 1, -1	349.4 (± 31.6)	34.3 (± 2.8)	9.8 (± 0.6)	13.9	23.8 (1.0)	*bd*	*nd*
17	-1, 1, -1, 1, -1	138.2 (± 3.9)	77.5 (± 11.0)	9.9 (± 1.3)	13.9	23.8 (1.0)	0.2	*nd*
18	1, 1, -1, 1, -1	383.9 (± 69.5)	75.8 (± 10.3)	10.2 (± 0.6)	13.9	23.8 (1.0)	0.1	*nd*
19	-1, -1, 1, 1, -1	148.7 (± 4.7)	31.2 (± 1.6)	23.4 (± 3.6)	13.9	23.8 (1.0)	*bd*	*nd*
20	1, -1, 1, 1, -1	356.1 (± 34.4)	33.3 (± 0.7)	23.1 (± 4.0)	13.9	23.8 (1.0)	*bd*	*nd*
21	-1, 1, 1, 1, -1	147.6 (± 8.1)	72.9 (± 7.6)	22.1 (± 4.2)	13.9	23.8 (1.0)	0.1	*nd*
22	1, 1, 1, 1, -1	359.3 (± 43.8)	76.8 (± 11.4)	23.7 (± 4.6)	13.9	23.8 (1.0)	*bd*	*nd*
23	-1, -1, -1, -1, 1	149.6 (± 5.9)	32.0 (± 2.1)	9.7 (± 1.0)	5.8	35.7 (0.4)	0.8	*nd*
24	1, -1, -1, -1, 1	407.5 (± 26.6)	34.6 (± 2.6)	9.2 (± 1.2)	5.8	35.7 (0.4)	*bd*	*nd*
25	-1, 1, -1, -1, 1	140.6 (± 6.2)	83.6 (± 4.9)	9.8 (± 1.3)	5.8	35.7 (0.4)	0.1	*nd*
26	1, 1, -1, -1, 1	423.3 (± 30.1)	82.7 (± 3.4)	9.6 (± 1.2)	5.8	35.7 (0.4)	0.2	*nd*
27	-1, -1, 1, -1, 1	156.2 (± 2.8)	32.0 (± 0.8)	24.2 (± 2.8)	5.8	35.7 (0.4)	*bd*	*nd*
28	1, -1, 1, -1, 1	371.1 (± 19.4)	33.5 (± 0.5)	24.7 (± 2.4)	5.8	35.7 (0.4)	*bd*	*nd*
29	-1, 1, 1, -1, 1	158.3 (± 2.7)	78.8 (± 1.7)	23.2 (± 3.0)	5.8	35.7 (0.4)	*bd*	*nd*
30	1, 1, 1, -1, 1	411.2 (± 8.1)	81.3 (± 6.9)	24.4 (± 3.9)	5.8	35.7 (0.4)	*bd*	*nd*
31	-1, -1, -1, 1, 1	138.4 (± 17.1)	29.3 (± 4.8)	8.8 (± 1.9)	13.9	35.7 (0.4)	*bd*	*nd*
32	1, -1, -1, 1, 1	350.1 (± 30.9)	33.3 (± 3.8)	9.0 (± 1.5)	13.9	35.7 (0.4)	*bd*	*nd*
33	-1, 1, -1, 1, 1	132.3 (± 2.0)	73.6 (± 14.8)	9.1 (± 2.0)	13.9	35.7 (0.4)	*bd*	*nd*

34	1, 1, -1, 1, 1	376.7 (± 76.7)	71.4 (± 14.8)	9.3 (± 1.6)	13.9	35.7 (0.4)	*bd*	*nd*
35	-1, -1, 1, 1, 1	160.6 (± 7.1)	31.5 (± 1.3)	21.5 (± 5.5)	13.9	35.7 (0.4)	*bd*	*nd*
36	1, -1, 1, 1, 1	352.8 (± 37.7)	32.0 (± 2.0)	21.0 (± 6.1)	13.9	35.7 (0.4)	*bd*	*nd*
37	-1, 1, 1, 1, 1	133.6 (± 22.1)	65.0 (± 15.5)	18.5 (± 7.8)	13.9	35.7 (0.4)	*bd*	*nd*
38	1, 1, 1, 1, 1	344.0 (± 59.1)	70.2 (± 18.0)	19.1 (± 9.1)	13.9	35.7 (0.4)	*bd*	*nd*
39	α, 0, 0, 0, 0	575.0 (± 6.6)	51.0 (± 4.2)	15.1 (± 3.3)	9.9	29.9 (0.6)	*bd*	*nd*
40	0, α, 0, 0, 0	274.5 (± 0.2)	106.9 (± 7.2)	15.6 (± 3.8)	9.9	29.9 (0.6)	*bd*	*nd*
41[d]	0, 0, α, 0, 0	287.4 (± 7.4)	53.0 (± 6.3)	68.4 (± 12.7)	9.9	29.9 (0.6)	*bd*	*nd*
42	0, 0, 0, α, 0	245.2 (± 27.4)	50.2 (± 9.9)	14.2 (± 5.2)	30.0	29.9 (0.6)	0.1	*nd*
43	0, 0, 0, 0, α	205.4 (± 67.2)	40.4 (± 19.7)	11.4 (± 7.9)	10.1	46.1 (1.1)	*bd*	*nd*
44	-α, 0, 0, 0, 0	0.0 (± 0.0)	53.8 (± 4.1)	17.3 (± 2.7)	9.9	29.9 (0.6)	*bd*	*nd*
45	0, -α, 0, 0, 0	271.1 (± 8.1)	0.0 (± 0.0)	18.5 (± 2.2)	9.9	29.9 (0.6)	*bd*	*nd*
46	0, 0, -α, 0, 0	257.1 (± 11.8)	56.9 (± 3.5)	0.0 (± 0.0)	9.9	29.9 (0.6)	0.1	*nd*
47	0, 0, 0, -α, 0	324.4 (± 51.8)	60.1 (± 0.1)	18.9 (± 0.4)	1.0	29.9 (0.6)	23.1	*nd*
48	0, 0, 0, 0, -α	271.4 (± 1.2)	58.6 (± 1.6)	17.8 (± 1.5)	9.9	14.9 (1.1)	4.6	*nd*
49	n/a	249.3 (± 23.3)	51.6 (± 8.5)	15.2 (± 4.1)	30.0	14.9 (1.1)	0.4	*nd*
50	n/a	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	10	29.9 (0.6)	84.2	80.6
51	n/a	0.6 (1.1)	0.1 (0.3)	0.0 (0.0)	30	29.9 (0.6)	81.6	79.1
52	n/a	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	10	14.9 (1.1)	83.3	83.3
53	n/a	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	30	14.9 (1.1)	83.2	81.7
54	n/a	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	6	35.7 (0.4)	84.6	82.5
55	n/a	1.8 (2.5)	0.4 (0.6)	0.1 (0.3)	14	35.7 (0.4)	82.5	79.1
56	n/a	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	6	23.8 (1.0)	84.0	77.6
57	n/a	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	14	23.8 (1.0)	83.6	81.5

[a] Coded factors are based on the first central composite design (CCD) for factors in the following order: *n*-butyric acid, *n*-valeric acid, *n*-caproic acid, exposure time, and temperature. Treatments 49-57 do not have coded values because they were added treatments that were not part of the CCD.

[b] Values shown are means. Values in parentheses give the range of values when there are only two measurements (carboxylic acid concentrations for Treatments 1-49; shown as ±) or the standard deviation for all others.

[c] *bd* indicates that percent viability was below detection. For data analysis, percent viability was assumed to equal the detection limit. *nd* indicates that a replicate was not performed for that treatment.

[d] Treatment 41 was excluded from data analysis because an error caused a very high concentration on *n*-caproic acid, and the extreme value interfered during model fitting.

Table A3.2 Summary of treatment conditions and results for Group 2.

Treat-ment #	Coded factors [a]	*n*-Butyric acid (mM) [b]	*n*-Valeric acid (mM) [b]	*n*-Caproic acid (mM) [b]	Exposure time (d)	Temp (°C) [b]	Viability 1 (%) [c]	Viability 2 (%) [c]
1	0, 0, 0, 0, 0	120.1 (± 0.2)	25 (± 0.8)	7.2 (± 0.3)	9.8	30.0 (0.5)	0.2	*nd*
2	0, 0, 0, 0, 0	122 (± 1.7)	25.9 (± 0.1)	7.4 (± 0.1)	9.8	30.0 (0.5)	*bd*	*nd*
3	0, 0, 0, 0, 0	124.1 (± 3.8)	26 (± 0.2)	7.5 (± 0)	9.8	30.0 (0.5)	*bd*	*nd*
4	0, 0, 0, 0, 0	124.8 (± 4.5)	26 (± 0.2)	7.4 (± 0.1)	9.8	30.0 (0.5)	1.4	*nd*
5	0, 0, 0, 0, 0	122.1 (± 1.8)	25.8 (± 0)	7.4 (± 0.1)	9.8	30.0 (0.5)	0.1	*nd*
6	0, 0, 0, 0, 0	124.6 (± 4.3)	26.3 (± 0.5)	7.5 (± 0)	9.8	30.0 (0.5)	0.6	*nd*
7	-1, -1, -1, -1, -1	67.5 (± 4.7)	15.6 (± 0.1)	4.3 (± 0.1)	5.9	26.1 (0.6)	78.9	*nd*
8	1, -1, -1, -1, -1	177.4 (± 6.8)	15.2 (± 2)	4 (± 0.1)	5.9	26.1 (0.6)	38.5	*nd*
9	-1, 1, -1, -1, -1	81.6 (± 4.2)	37 (± 1.1)	4.8 (± 0.1)	5.9	26.1 (0.6)	69.6	*nd*
10	1, 1, -1, -1, -1	179.7 (± 6.8)	34.2 (± 1.4)	4.1 (± 0.1)	5.9	26.1 (0.6)	7.4	*nd*
11	-1, -1, 1, -1, -1	78.4 (± 3.6)	15.7 (± 1.1)	12.4 (± 1)	5.9	26.1 (0.6)	64.5	*nd*
12	1, -1, 1, -1, -1	167.3 (± 3.5)	14.6 (± 1.1)	11 (± 0.5)	5.9	26.1 (0.6)	5.5	*nd*
13	-1, 1, 1, -1, -1	68.4 (± 1.9)	35.3 (± 1.3)	10.7 (± 1)	5.9	26.1 (0.6)	67.4	*nd*
14	1, 1, 1, -1, -1	163.3 (± 6.3)	35.5 (± 2.8)	9.8 (± 0.3)	5.9	26.1 (0.6)	10.9	*nd*
15	-1, -1, -1, 1, -1	69.8 (± 2.4)	14.8 (± 0.9)	4.2 (± 0.2)	13.6	25.9 (0.5)	64.4	*nd*
16	1, -1, -1, 1, -1	169.8 (± 0.8)	13.8 (± 0.6)	3.9 (± 0)	13.6	25.9 (0.5)	0.1	*nd*
17	-1, 1, -1, 1, -1	76.1 (± 1.3)	35.4 (± 0.5)	4.9 (± 0)	13.6	25.9 (0.5)	9.6	*nd*
18	1, 1, -1, 1, -1	167.8 (± 5.1)	33.6 (± 0.8)	4.1 (± 0.1)	13.6	25.9 (0.5)	*bd*	*nd*
19	-1, -1, 1, 1, -1	77.1 (± 4.9)	15.4 (± 1.4)	12.5 (± 0.9)	13.6	25.9 (0.5)	5.3	*nd*
20	1, -1, 1, 1, -1	170.4 (± 0.4)	13.6 (± 0.1)	10.2 (± 0.3)	13.6	25.9 (0.5)	*bd*	*nd*
21	-1, 1, 1, 1, -1	69 (± 1.3)	35.1 (± 1.5)	10.9 (± 0.8)	13.6	25.9 (0.5)	0.3	*nd*
22	1, 1, 1, 1, -1	166.7 (± 9.7)	33.8 (± 1.1)	9.5 (± 0)	13.6	25.9 (0.5)	*bd*	*nd*
23	-1, -1, -1, -1, 1	68.5 (± 3.7)	15.7 (± 0)	4.1 (± 0.3)	5.9	35.9 (0.8)	47.9	*nd*
24	1, -1, -1, -1, 1	165.8 (± 4.8)	13.4 (± 0.2)	3.7 (± 0.2)	5.9	35.9 (0.8)	*bd*	*nd*
25	-1, 1, -1, -1, 1	80.2 (± 2.8)	36.8 (± 0.9)	5 (± 0.1)	5.9	35.9 (0.8)	0.1	*nd*
26	1, 1, -1, -1, 1	170.1 (± 2.8)	33.8 (± 1)	3.9 (± 0.3)	5.9	35.9 (0.8)	*bd*	*nd*
27	-1, -1, 1, -1, 1	78.5 (± 3.5)	15.3 (± 1.5)	11.6 (± 1.8)	5.9	35.9 (0.8)	0.2	*nd*
28	1, -1, 1, -1, 1	172.2 (± 1.4)	14.8 (± 1.3)	10.2 (± 0.3)	5.9	35.9 (0.8)	*bd*	*nd*
29	-1, 1, 1, -1, 1	69.4 (± 0.9)	35.2 (± 1.4)	10.3 (± 1.4)	5.9	35.9 (0.8)	*bd*	*nd*
30	1, 1, 1, -1, 1	154.3 (± 2.7)	33.2 (± 0.5)	8.9 (± 0.6)	5.9	35.9 (0.8)	*bd*	*nd*
31	-1, -1, -1, 1, 1	72 (± 0.2)	14.7 (± 1)	3.9 (± 0.5)	13.6	35.6 (0.7)	3.2	*nd*
32	1, -1, -1, 1, 1	158.2 (± 12.4)	12.4 (± 0.8)	3.4 (± 0.5)	13.6	35.6 (0.7)	0.2	*nd*
33	-1, 1, -1, 1, 1	73.9 (± 3.5)	33.2 (± 2.7)	4.3 (± 0.6)	13.6	35.6 (0.7)	*bd*	*nd*
34	1, 1, -1, 1, 1	170.9 (± 2)	32.5 (± 0.3)	3.7 (± 0.5)	13.6	35.6 (0.7)	*bd*	*nd*
35	-1, -1, 1, 1, 1	76.3 (± 5.7)	14.9 (± 1.9)	11.5 (± 1.9)	13.6	35.6 (0.7)	*bd*	*nd*
36	1, -1, 1, 1, 1	168 (± 2.8)	13.2 (± 0.3)	9.4 (± 1.1)	13.6	35.6 (0.7)	*bd*	*nd*
37	-1, 1, 1, 1, 1	69.7 (± 0.6)	34.5 (± 2.1)	10.1 (± 1.6)	13.6	35.6 (0.7)	*bd*	*nd*
38	1, 1, 1, 1, 1	163.9 (± 6.9)	31.9 (± 0.8)	8.3 (± 1.2)	13.6	35.6 (0.7)	*bd*	*nd*
39	α, 0, 0, 0, 0	222.9 (± 18.5)	24.6 (± 0.4)	7.8 (± 0.3)	9.8	30.0 (0.5)	*bd*	*nd*

40	0, α, 0, 0, 0	123.2 (± 1.3)	49.9 (± 1.7)	7.1 (± 0.4)	9.8	30.0 (0.5)	*bd*	*nd*
41	0, 0, α, 0, 0	122.7 (± 4)	24.7 (± 1)	14.7 (± 1.6)	9.8	30.0 (0.5)	*bd*	*nd*
42	0, 0, 0, α, 0	118.5 (± 1.8)	23.4 (± 2.4)	6.3 (± 1.2)	18.9	29.9 (0.4)	*bd*	*nd*
43	0, 0, 0, 0, α	118.2 (± 2.1)	23.4 (± 2.4)	5.9 (± 1.6)	9.8	44.0 (0.2)	*bd*	*nd*
44	-α, 0, 0, 0, 0	0 (± 0)	24.7 (± 0.5)	7.5 (± 0.2)	9.8	30.0 (0.5)	53.4	*nd*
45	0, -α, 0, 0, 0	135.7 (± 1.6)	0 (± 0)	6.8 (± 0)	9.8	30.0 (0.5)	5.5	*nd*
46	0, 0, -α, 0, 0	123.7 (± 3.5)	24.9 (± 0.6)	0 (± 0)	9.8	30.0 (0.5)	6.0	*nd*
47	0, 0, 0, -α, 0	125.1 (± 4.8)	26.9 (± 1.1)	7.7 (± 0.2)	0.8	31.4 (0.7)	78.2	*nd*
48	0, 0, 0, 0, -α	125.7 (± 5.4)	26.7 (± 0.9)	7.9 (± 0.4)	9.8	15.3 (0.8)	77.0	*nd*
49	0, 0, 0, 0, 0 (CCD 1)	227.8 (± 7.8)	43.4 (± 2.3)	11.4 (± 0.4)	9.8	30.0 (0.5)	*bd*	*nd*
50	0, 0, 0, 0, 0 (CCD 1)	231 (± 4.6)	42.7 (± 1.6)	11.2 (± 0.2)	9.8	30.0 (0.5)	*bd*	*nd*
51	0, 0, 0, 0, 0 (CCD 1)	232.4 (± 3.2)	42.6 (± 1.5)	10.9 (± 0)	9.8	30.0 (0.5)	*bd*	*nd*
52	0, 0, 0, 0, 0 (CCD 1)	236.9 (± 1.3)	43.4 (± 2.2)	11.3 (± 0.3)	9.8	30.0 (0.5)	*bd*	*nd*
53	0, 0, 0, 0, 0 (CCD 1)	234.8 (± 0.8)	42.5 (± 1.4)	11 (± 0)	9.8	30.0 (0.5)	*bd*	*nd*
54	0, 0, 0, 0, 0 (CCD 1)	227.6 (± 7.9)	42.1 (± 1)	10.9 (± 0)	9.8	30.0 (0.5)	*bd*	*nd*
55	n/a	0	0	0	9.8	30.0 (0.5)	82.2	82.9
56	n/a	0	0	0	5.9	26.1 (0.6)	77.6	82.1
57	n/a	0	0	0	13.6	25.9 (0.5)	83.1	80.6
58	n/a	0	0	0	5.9	35.9 (0.8)	77.8	80.7
59	n/a	0	0	0	13.6	35.6 (0.7)	53.1	38.7
60	n/a	0	0	0	9.8	44.0 (0.2)	*bd*	*bd*
61	n/a	0	0	0	9.8	15.3 (0.8)	81.6	80.3
62	n/a	0	0	0	0.8	31.4 (0.7)	81.1	*nd*
63	n/a	0	0	0	18.9	29.9 (0.4)	80.5	80.4

[a] Coded factors are based on the second CCD for Treatments 1-48. Treatments 49-54 are the repeated center treatment from the first CCD. Treatments 55-63 do not have coded values because they were not part of either CCD. Coded factors are given in the following order: *n*-butyric acid, *n*-valeric acid, *n*-caproic acid, exposure time, and temperature.

[b] Values shown are means. Values in parentheses give the range of values when there are only two measurements (carboxylic acid concentrations for Treatments 1-54; shown as ±) or the standard deviation for others.

[c] *bd* indicates that percent viability was below detection. For data analysis, percent viability was assumed to equal the detection limit. *nd* indicates that a replicate was not performed for that treatment.

Table A3. 3 Summary of treatment conditions and results for Group 3.

Treat-ment #	Coded factors [a]	*n*-Butyric acid (mM) [b]	*n*-Valeric acid (mM) [b]	*n*-Caproic acid (mM) [b]	Exposure time (d)	Temp (°C) [b]	Viability 1 (%) [c]	Viability 2 (%) [c]
1	-1, -1, -1, 0, -1	68.3 (0.5)	12.8 (0.2)	3.4 (0.2)	9.9	24.8 (0.2)	67.2	66.5
2	1, -1, -1, 0, -1	174.2 (4.3)	13 (0.3)	3.2 (0.1)	9.9	24.8 (0.2)	13.6	8.0
3	-1, 1, -1, 0, -1	72.3 (1)	31.7 (0.2)	4.1 (0.2)	9.9	24.8 (0.2)	36.7	45.7
4	1, 1, -1, 0, -1	172.1 (9.9)	31.8 (0.9)	3.4 (0.1)	9.9	24.8 (0.2)	2.2	0.9
5	-1, -1, 1, 0, -1	73.4 (1.4)	12.8 (0.2)	10 (0.3)	9.9	24.8 (0.2)	41.8	43.6
6	1, -1, 1, 0, -1	174.3 (7.2)	12.7 (0.5)	9 (0.5)	9.9	24.8 (0.2)	1.2	1.3
7	-1, 1, 1, 0, -1	70.1 (2.9)	31.9 (0.7)	8.9 (0.3)	9.9	24.8 (0.2)	10.4	10.2
8	1, 1, 1, 0, -1	178.1 (6.7)	31.8 (0)	8.2 (0.5)	9.9	24.8 (0.2)	*bd*	0.1
9	-1, -1, -1, n/a, 1	68.3 (1.4)	12.4 (0.2)	4.1 (0.2)	3.0	36.8 (0.1)	73.9	70.9
10	1, -1, -1, n/a, 1	175.1 (4.1)	12.9 (0.5)	3.1 (0.3)	3.0	36.8 (0.1)	15.0	11.7
11	-1, 1, -1, n/a, 1	71.9 (0.8)	31.4 (0.6)	4 (0.3)	3.0	36.8 (0.1)	38.5	29.9
12	1, 1, -1, n/a, 1	170.8 (8.2)	31.3 (0.3)	3.3 (0.2)	3.0	36.8 (0.1)	*bd*	*bd*
13	-1, -1, 1, n/a, 1	70.9 (2.2)	12.5 (0.5)	9.5 (0.7)	3.0	36.8 (0.1)	14.0	23.6
14	1, -1, 1, n/a, 1	169.7 (1)	12.6 (0.6)	8.8 (0.7)	3.0	36.8 (0.1)	*bd*	*bd*
15	-1, 1, 1, n/a, 1	68.3 (0.8)	30.9 (0.5)	8.5 (0.6)	3.0	36.8 (0.1)	0.6	0.6
16	1, 1, 1, n/a, 1	176.2 (5.9)	31.4 (0.7)	8 (0.7)	3.0	36.8 (0.1)	*bd*	*bd*
17	0, 0, 0, n/a, -1	112.1 (0.7)	22.2 (0.3)	6.3 (0.1)	3.1	24.9 (0.2)	70.7	67.7
18	0, 0, 0, -1, -1	116.9 (5.9)	22.6 (0.3)	6.2 (0.1)	6.1	24.8 (0.2)	42.5	47.2
19	0, 0, 0, 0, -1	117.6 (6.1)	22.6 (0.3)	6.2 (0.1)	9.9	24.8 (0.2)	9.4	11.4
20	0, 0, 0, n/a, 0	112.2 (1.5)	22 (0.4)	6.2 (0.2)	3.1	27.8 (0.5)	62.7	60.7
21	0, 0, 0, n/a, 1	116.5 (4.5)	22.7 (0.4)	6.2 (0.2)	3.0	36.8 (0.1)	1.0	1.7
22	-1, -1, -1, n/a, 0	65.8 (3.1)	12.6 (0.4)	3.4 (0.2)	3.1	27.8 (0.5)	72.5	71.0
23	1, -1, -1, n/a, 0	166.1 (8.7)	12.8 (0.5)	3.2 (0.2)	3.1	27.8 (0.5)	65.1	62.9
24	-1, 1, -1, n/a, 0	69 (3.5)	30.9 (1.1)	4.1 (0.2)	3.1	27.8 (0.5)	69.5	71.6
25	1, 1, -1, n/a, 0	160.8 (4.6)	30.6 (0.6)	3.4 (0.1)	3.1	27.8 (0.5)	45.4	60.8
26	-1, -1, 1, n/a, 0	69 (4.5)	12.4 (0.6)	9.8 (0.4)	3.1	27.8 (0.5)	66.0	65.9
27	1, -1, 1, n/a, 0	164.8 (5.1)	12.7 (0.5)	8.9 (0.6)	3.1	27.8 (0.5)	31.9	25.1
28	-1, 1, 1, n/a, 0	65 (3.1)	30.3 (1.2)	8.7 (0.4)	3.1	27.8 (0.5)	66.3	57.2
29	1, 1, 1, n/a, 0	167.9 (5.6)	31.1 (0.9)	8.2 (0.6)	3.1	27.8 (0.5)	5.3	4.2
30	-1, -1, -1, -1, 0	68.7 (0.6)	12.8 (0.2)	3.4 (0.2)	6.1	27.3 (0.3)	68.7	68.3
31	1, -1, -1, -1, 0	178.6 (10.7)	13.1 (0.5)	3.3 (0.1)	6.1	27.3 (0.3)	14.4	22.8
32	-1, 1, -1, -1, 0	72.9 (1.4)	31.3 (0.8)	4 (0.2)	6.1	27.3 (0.3)	68.2	45.3
33	1, 1, -1, -1, 0	170.2 (7.5)	31.2 (0.3)	3.4 (0.1)	6.1	27.3 (0.3)	0.6	0.2
34	-1, -1, 1, -1, 0	72.2 (1.1)	12.6 (0.4)	9.7 (0.5)	6.1	27.3 (0.3)	38.9	36.5
35	1, -1, 1, -1, 0	175.6 (7.6)	12.7 (0.5)	9 (0.4)	6.1	27.3 (0.3)	*bd*	0.2
36	-1, 1, 1, -1, 0	86.6 (37.2)	34.4 (6.3)	11.2 (4.6)	6.1	27.3 (0.3)	5.8	3.6
37	1, 1, 1, -1, 0	178.7 (7.5)	31.7 (0.1)	8.3 (0.4)	6.1	27.3 (0.3)	*bd*	*bd*
38	-1, -1, -1, -1, -1	69 (1.1)	12.8 (0.1)	3.4 (0.1)	6.1	27.3 (0.3)	68.2	61.0
39	-1, -1, -1, -1, 1	68 (0.6)	12.5 (0.5)	3.6 (0.1)	6.1	36.9 (0.2)	57.0	60.7
40	0, 0, 0, 0, 0	119.4 (7.9)	22.8 (0.5)	6.2 (0.2)	9.9	27.3 (0.4)	0.3	0.3

[a] Coded factors are based on the second CCD where applicable. They are given in the following order: *n*-butyric acid, *n*-valeric acid, *n*-caproic acid, exposure time, and temperature.
[b] Values shown are means with standard deviations in parentheses.
[c] *bd* indicates that percent viability was below detection. For data analysis, percent viability was assumed to equal the detection limit.

Table A3.4 Summary of treatment conditions and results for Group 4. Coded factors are not given because treatments were not based on either CCD.

Treat-ment #	*n*-Butyric acid (mM) [a]	*n*-Valeric acid (mM) [a]	*n*-Caproic acid (mM) [a]	Exposure time (d)	Temp (°C) [a]	Viability 1 (%) [c]	Viability 2 (%) [c]
1	243.2 (7.5)	12.1 (0.2)	26.4 (0.1)	1.1	23.1 (0.3)	40.3	43.1
2	250.1 (15.7)	12.2 (0.4)	26.3 (0.6)	3.0	22.9 (0.3)	0.3	0.2
3	247.6 (13.5)	12.2 (0.4)	26.3 (0.4)	5.0	23.0 (0.3)	*bd*	*bd*
4	244.3 (6.7)	12.1 (0.2)	26.2 (0.4)	7.1	23.0 (0.3)	*bd*	*bd*
5	238.6 (n/a)	12.0 (n/a)	26.3 (n/a)	12.0	23.0 (0.3)	*bd*	*bd*
6	246.8 (12.8)	12.2 (0.3)	26.3 (0.2)	1.1	31.5 (0.2)	*bd*	*bd*
7	247.2 (11.9)	12 (0.1)	25.5 (0.9)	3.0	31.5 (0.1)	0.2	*bd*
8	240.1 (2.5)	11.9 (0.2)	25.2 (1.3)	5.0	31.5 (0.2)	*bd*	*bd*
9	242.1 (6.4)	11.8 (0.3)	24.7 (2.0)	7.1	31.4 (0.2)	0.3	*bd*
10	241.9 (3.9)	12 (0.1)	25.8 (0.7)	1.1	36.8 (0)	*bd*	*bd*
11	245.4 (8.0)	12 (0.2)	25.4 (1.1)	2.1	36.9 (0.4)	*bd*	*bd*
12	239.7 (1.8)	11.8 (0.2)	24.4 (2.2)	4.0	36.9 (0.4)	*bd*	*bd*
13	243.1 (5.4)	11.7 (0.3)	23.7 (2.9)	6.0	37.2 (0.7)	*bd*	*bd*
14 [d]	173 (4.5)	33.1 (0.9)	10.0 (0.4)	3.0	31.5 (0.1)	*bd*	0.3
15 [e]	175.8 (3.6)	33.6 (0.3)	10.0 (0.4)	6.0	31.4 (0.2)	*bd*	*bd*

[a] Values shown are means with standard deviations in parentheses.
[b] *bd* indicates that percent viability was below detection. For data analysis, percent viability was assumed to equal the detection limit.
[c] Treatment 14 is a replicate of Treatment 29 in Group 3.
[d] Treatment 15 is a replicate of Treatment 37 in Group 3.

Table A3.5 Model coefficients and corresponding statistics from the second CCD.

Predictor	Coefficient	Standard error	*p*-value	Significance [a]
Intercept	843	102	6.64×10^{-9}	***
Bu	-2.43	0.368	4.42×10^{-7}	***
Va	-3.44	1.81	0.0686	NS
Ca	-13.2	5.99	0.0364	*
Ti	-31.2	4.94	9.27×10^{-7}	***
Te	-24.6	3.79	5.88×10^{-7}	***
Bu^2	0.00180	6.78×10^{-4}	0.0130	*
Va^2	0.00502	0.0140	0.722	NS
Ca^2	0.0535	0.159	0.739	NS
Ti^2	0.533	0.107	3.18×10^{-5}	***
Te^2	0.195	0.0434	1.16×10^{-4}	***
Bu ∗ Va	0.00747	0.00418	0.0850	NS
Bu ∗ Ca	0.0235	0.0132	0.0858	NS
Bu ∗ Ti	0.0337	0.0107	0.00384	**
Bu ∗ Te	0.0352	0.0084	2.86×10^{-4}	***
Va ∗ Ca	0.107	0.0625	0.0980	NS
Va ∗ Ti	0.00969	0.0501	0.848	NS
Va ∗ Te	0.0346	0.0397	0.391	NS
Ca ∗ Ti	0.119	0.156	0.451	NS
Ca ∗ Te	0.151	0.124	0.233	NS
Ti ∗ Te	0.400	0.103	5.79×10^{-4}	***

[a] *p*-value< 0.001 ***; *p*-value< 0.01 **; *p*-value< 0.05 *; *p*-value ≥0.05 NS

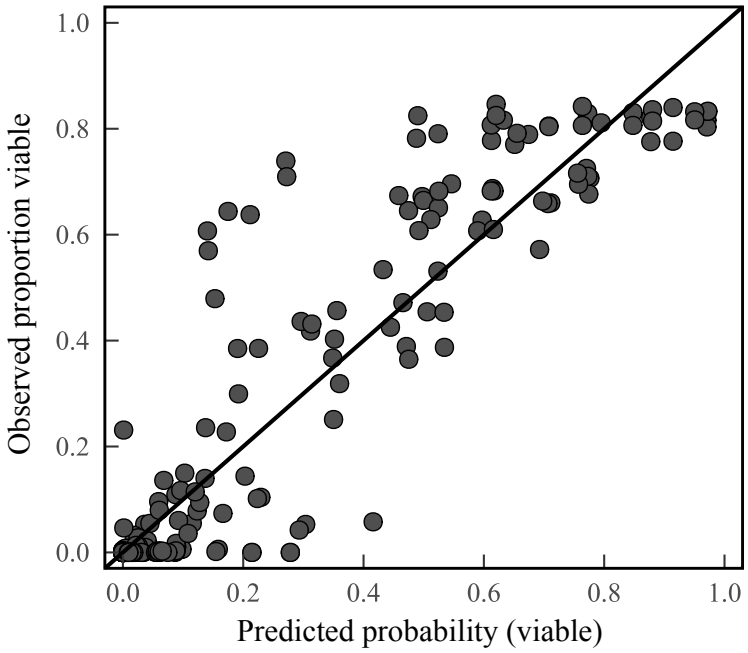

Figure A3.1 Observed and predicted values for the selected logistic regression model containing linear terms and two-way interactions for all five factors. Observations are from all treatments in Groups 1-4.

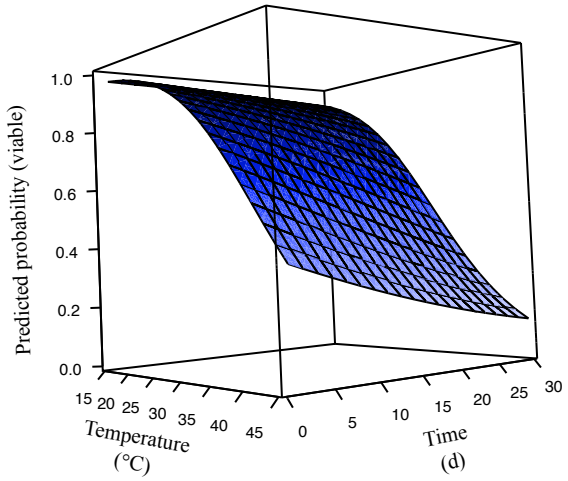

Figure A3.2 Response surface plots for the selected logistic regression model containing linear terms and two-way interactions for all five factors. Surface shows the effect of time and temperature on *Ascaris* viability when *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid concentrations are 0 mM.

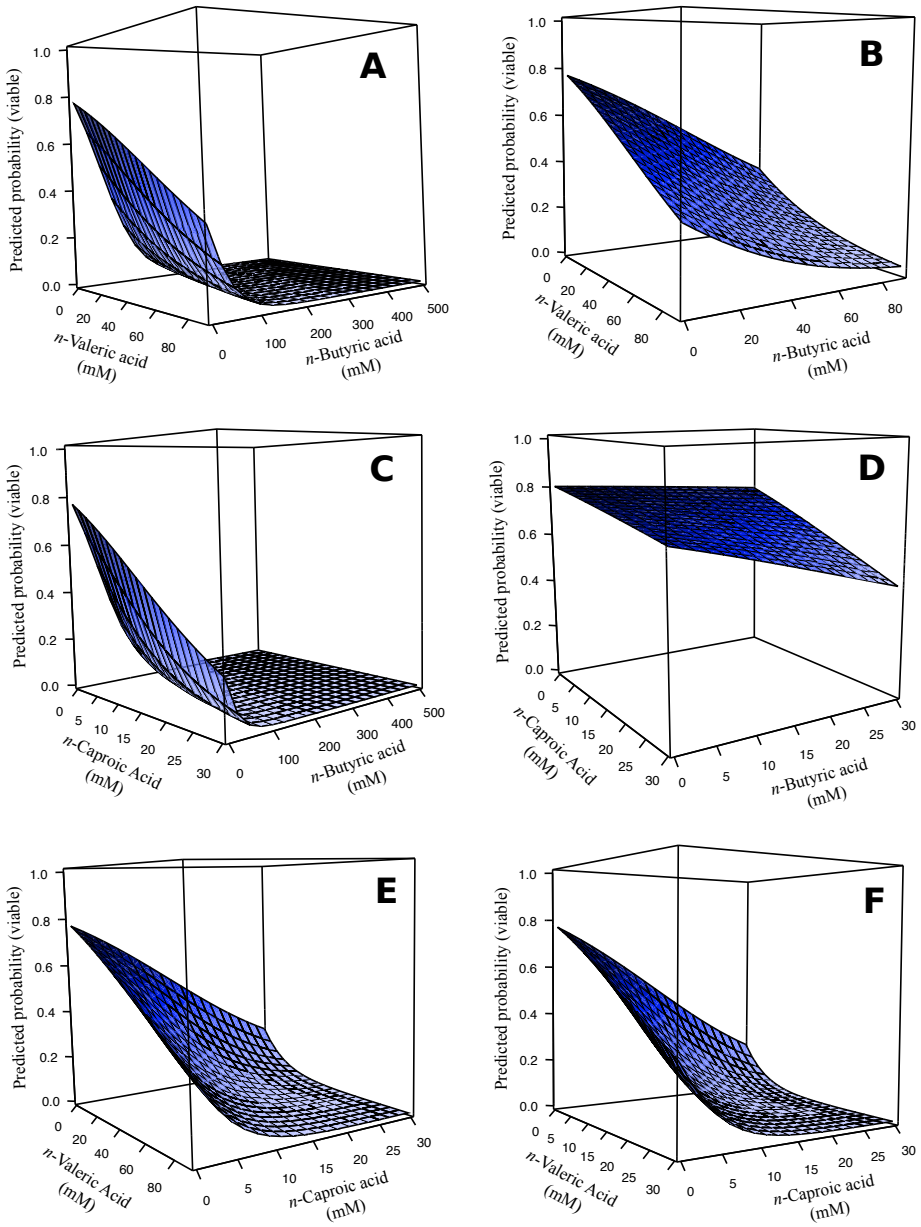

Figure A3.3 Response surface plots for the selected logistic regression model containing linear terms and two-way interactions for all five factors. Surfaces compare the effects of each carboxylic acid. *n*-Butyric acid and *n*-valeric acid are compared in panels **A** and **B**. *n*-Butyric acid and *n*-caproic acid are compared in panels **C** and **D**. *n*-Valeric acid and *n*-caproic acid are compared in panels **E** and **F**. Panels **A**, **C**, and **E** show each carboxylic acid at the full range of concentrations tested. In Panels **B**, **D**, and **F** the concentrations are shown on the same scale to better visualize the effect a unit increase in concentration of each acid. For all panels, time and temperature are held constant at the center point of the second CCD (time= 10 d and temperature= 30.0 °C). The carboxylic acid not shown in each panel is set equal to 0 mM.

APPENDIX 4

SUPPLEMENTARY INFORMATION FOR CHAPTER 6

Figure A4.1 Photos of batch fermentation media bottles used for Experiments 1 and 2.

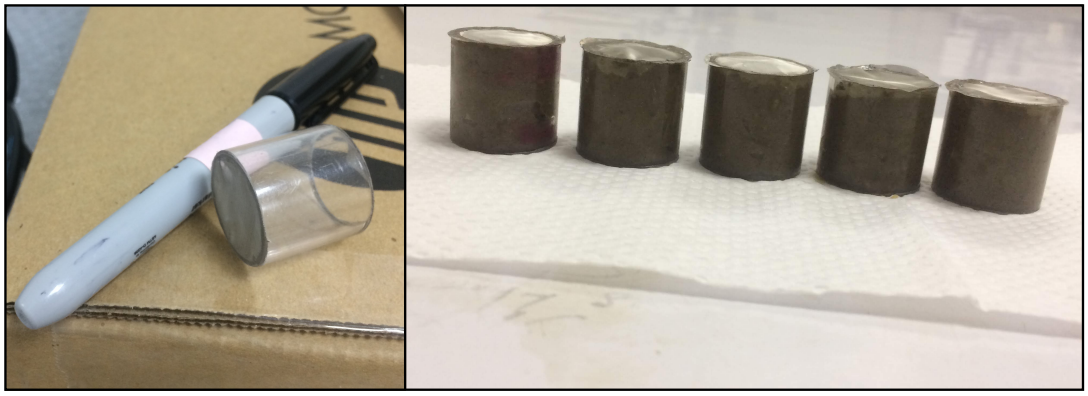

Figure A4.2 Photos of chambers used to contain *A. suum* eggs in Experiments 2 and 3. Photo on left shows an empty chamber with one end open for loading. Marker is shown for size comparison. The photo on the right shows chambers after they were loaded with treatment mixtures and *A. suum* eggs, and both ends were sealed with mesh.

Figure A4.3 Plastic barrels with screw-top lids used for field-scale trial during Experiment 3.

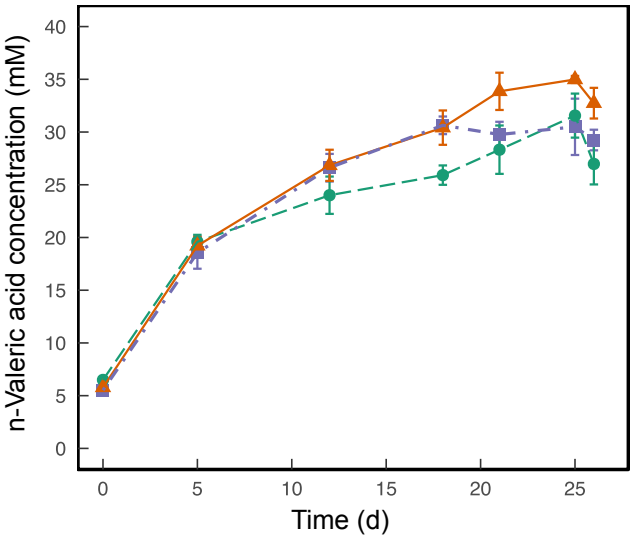

Figure A4.4 Average concentrations of *n*-valeric acid found in triplicate batch fermentation media bottles that were started with inoculum from a semi-continuous chain-elongation-bioreactor (●); inoculum from rumen contents of a cow (■); and no inoculum (▲). Error bars indicate standard deviations of the measured concentrations.

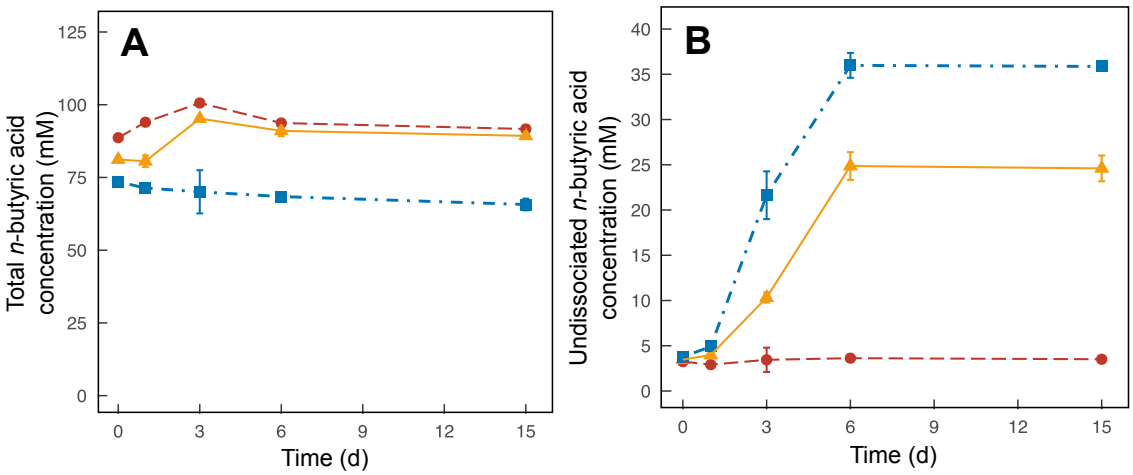

Figure A4.5 Average total *n*-butyric acid concentration (undissociated plus dissociated) (A) and undissociated *n*-butyric acid concentration (B) found in duplicate batch fermentation media bottles with banana mash added at 0 % (m/m) (●), 10 % (m/m) (▲), and 20 % (m/m) (■). Error bars indicate the range of the measured values between the duplicate media bottles.

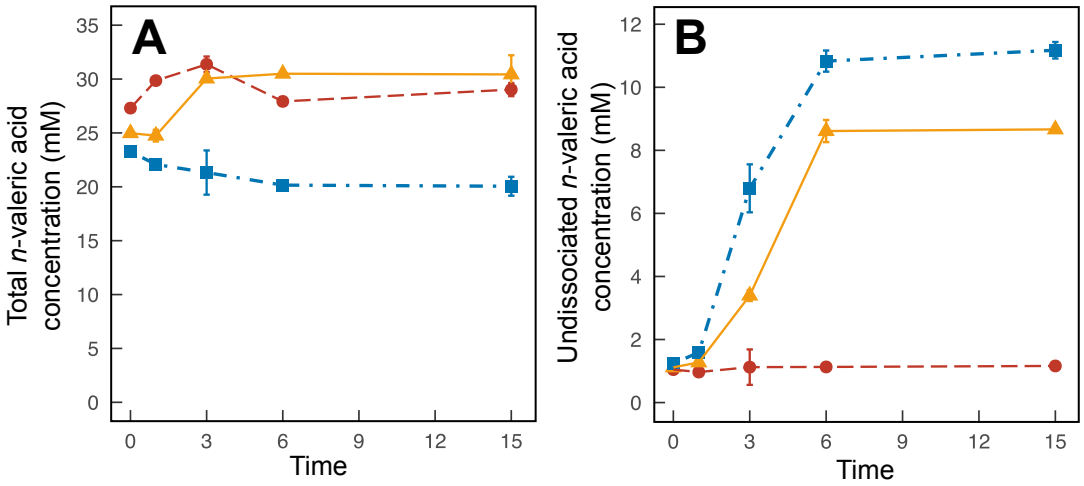

Figure A4.6 Average total *n*-valeric acid concentration (undissociated plus dissociated) (A) and undissociated *n*-valeric acid concentration (B) found in duplicate batch fermentation media bottles with banana mash added at 0 % (m/m) (●), 10 % (m/m) (▲), and 20 % (m/m) (■). Error bars indicate the range of the measured values between the duplicate media bottles.

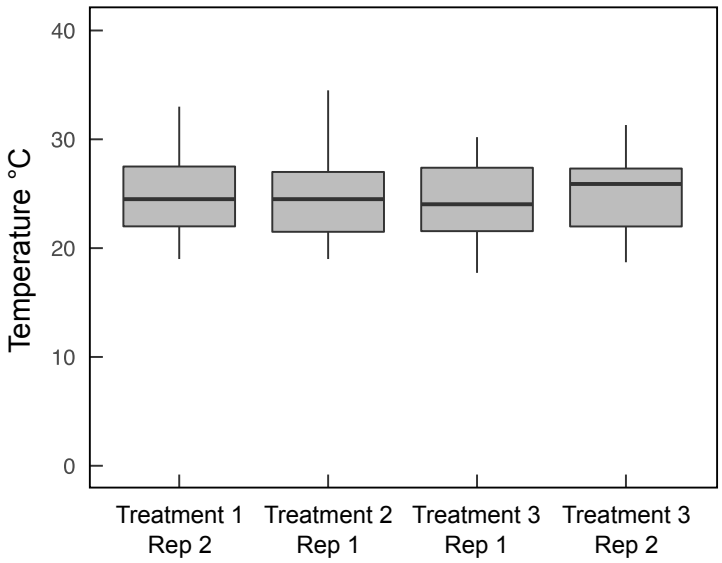

Figure A4.7 Boxplot of temperature recordings for the duration of the field-scale trial during Experiment 3.

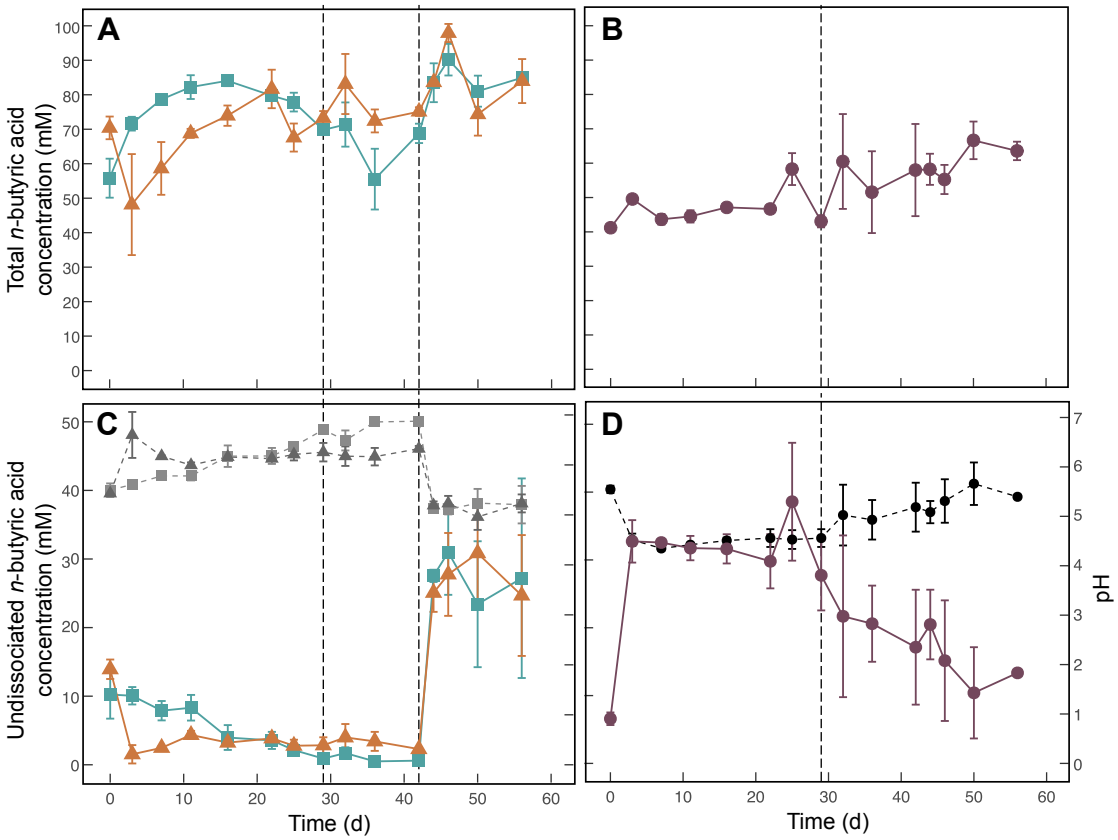

Figure A4.8 Total *n*-butyric acid concentrations (A and B), undissociated *n*-butyric acid concentrations (C and D), and pH (C and D) for field-scale trial during Experiment 3. For C and D, undissociated *n*-butyric acid concentrations (solid colored lines) are plotted against the left axis, and pH (dashed, gray-scale lines) is plotted against the right axis. Treatments 1 (■) and 2 (▲), which were conducted in two stages are shown on the left (A and C). Treatment 3 (●), which had banana waste mixed with UDDT-SW at initial startup is shown on the right (B and D). Error bars show the range between duplicate barrels. The dashed vertical line at Day 29 indicates the time that barrels were moved into the greenhouse, and the average temperature increased from 22.1°C to 26.9°C. The dashed vertical line at Day 42 in A and C indicates the time that banana waste was added to Treatments 1 and 2.

APPENDIX 5

STANDARD OPERATING PROCEDURE FOR MEASURING CARBOXYLIC ACID CONCENTRATIONS USING GAS CHROMATOGRAPHY

Sample Preparation and Storage:

1. Centrifuge samples and filter (0.2 μm) to remove any suspended solids.
2. Undiluted samples (no formic acid added) may be stored in 4° C refrigerator or freezer for subsequent analysis. Depending on the sample, it may need to be centrifuged and/or filtered again after thawing. If there is any possibility of suspended solids, make sure to refilter or centrifuge.
3. Each sample vial will contain: 3 mM 2-ethylbutytric acid (internal standard), sample (diluted to < 7 mM for individual VFA of interest), and 2% formic acid (to raise total volume to 1 mL or 500μL)
    a. Add appropriate volume of sample to obtain < 7 mM of the specific VFA of interest. If measuring multiple VFAs, multiple dilutions may be needed.
    b. Add stock internal standard. There should be a stock of 30 mM 2-ethylbutryic acid diluted in 2% formic acid. If using 1 mL total volume in the vial, add 100 μL of this stock solution. If using 500 μL total volume, add 50 μL stock solution.
    c. Add 2% formic acid to bring total volume to either 500 μL or 1 mL.
    d. Close vial immediately after adding formic acid.

    *** Note: Final pH of sample must be approximately 2 in order to push the fatty acids in the sample into the undissociated form. Depending on the alkalinity of the sample, this will restrict options for choosing dilutions. If sample contains very low concentrations of VFAs, a more concentrated formic acid solution can be used for dilutions to allow lower pH at higher sample concentration.

Standard Preparation:

The stock solution of volatile fatty acids (stored in the refrigerator in B68) contains 10 mM each of: formic, acetic, propionic, isobutyric, n-butyric, isovaleric, n-valeric, isocaproic, n-caproic, and n-heptanoic acids.

Make at least 5 standards of varying dilutions to create a standard curve. Typically a curve is created with 1, 2, 3, 5, and 7 mM. Choose appropriate concentrations based on expected concentrations in samples. If sample concentrations are low, the GC can detect accurately down to about 0.1 mM, but the standard curve needs to include points in this range.

Make additional "check standards" of known concentration to mix in the run with samples. This provides quality control because the measured concentrations can be compared to the known concentrations. (See below in "Loading Samples" for more details.)

Make a couple blank vials of only 2% formic acid. These will be run as the first vial, after the set of standards, after every 5 samples, and as the final vial. You can have the same autosampler return to the same vial multiple times.

Start-up:

1. **FIRST** turn on the compressed air, helium, and hydrogen cylinders by opening the main cylinder valves.
2. Add name, date, and planned number of samples to the logbook.
3. If the septum has not been changed for over 50 injections (recorded in logbook), change it now.
    a. Remove the injector tower by lifting straight up off of the support rod. Place injector tower on the top of GC 2 or on shelf.
    b. Remove the nut (green in photo) at injection site.
    c. Remove green septum and discard. Septum may be lodged inside the nut.
    d. Place in new septum and replace nut. Only finger tighten the nut. It does not need to be overly tight.
    e. Replace injector tower by aligning metal support rod with appropriate port on the bottom of the tower.
4. If the glass injector sleeve has not been replaced for over 150 injections (recorded in log book), replace now. If acetate measurements are important, check the glass injector sleeve for contamination even if it has been recently replaced. If the acetate standard curve levels off at higher concentrations, this is most often due to a dirty injector sleeve or dirty column.
    a. Remove injector tower.
    b. Open top door of the GC that flips upward.
    c. Use wide wrench located on bench next to the GCs to loosen the larger nut underneath septum nut.
    d. Squeeze pointed forceps together and insert into injection port.
    e. Allow forceps to expand and pull straight up to remove glass injector sleeve.
    f. If contamination is visible, place used injector sleeve in glass Nalgene bottle labeled 25% H_2SO_4 and containing other glass sleeves. Use forceps to remove clean sleeve from bottle.
    g. Rinse new sleeve with ethanol followed by DI water.
    h. Dry with compressed air from the lab bench.
    i. Use forceps to drop new, clean sleeve into injection port with flared end at the top.
    j. Tighten nut with wrench. Again, does not need to be overly tight but this one is a bit harder than the septum nut.

k. Replace injector tower.

5. Turn the switch on the bottom left corner of the front face of the machine on.
6. Turn on the communication module (box located to the left of the GC).
7. On the Windows 98 computer, open "Instrument 1 (online)" from the desktop. If "Instrument 1 (offline)" is already open, close that before opening the online program.
8. After Chemstation loads on the GC, check that the oven, front injector, and front detector are all on and temperatures are set to 70, 200, and 275° C, respectively.
9. Empty waste vials and methanol (Solvent A) from the injection tower into the GC waste bottle (stored under the right-hand fume hood when back is to the windows).
10. Refill Solvent A with fresh methanol (found under the right-hand fume hood in B68A).
11. Empty and refill Solvent B vial with DI water.

***Both Solvent A and Solvent B should be filled completely! The needle only lowers down to about the 2 mL mark, so if the vials contain less than 2 mL of liquid, the needle will not be cleaned.

12. Remove and clean needle.
    a. Open door on the injection tower.
    b. Swing open the tab located about halfway up the syringe barrel that is holding the needle in place.
    c. Unscrew the nut holding the plunger and slide nut up.
    d. Pull needle forward from point D on the photo and then lift up to remove. (Otherwise the needle tip will get caught on the bottom stand.)
    e. Remove plunger and clean the plunger and syringe barrel using ethanol, soap, and DI water. Check that the plunger moves smoothly through the barrel.
    f. Fill a small beaker with DI water and check that the needle takes up and dispels water properly.
    g. Replace needle properly. Hold needle at an angle and align needle tip with position on gray stand. Then slide into position at point D. Rescrew the nut a point C and replace the tab at point B.

Loading Samples (this can be performed while waiting for the GC to warm up)

1. Load your samples into the autosampler trays. Ensure that the "Vial 1" position on the tray is aligned with the "1" position on the autosampler.
2. Include a blank vial containing only 2% formic acid as the first vial, after the set of standards, after every 5 samples, and as the final vial.
3. Approximately every 10 vials add one of the "check standards" created during "**Standard Preparation**".
4. As best as can be predicted, try to load the samples in order from most dilute to most concentrated.
5. For quality control, recommended run length is a maximum of 50 vials including blanks and standards. After a long period of time, the GC measurements tend to fluctuate.

Loading Sequence on the Computer

1. From the "Online" computer program (Instrument 1 or 3 depending on which machine is being used), click "New Sequence" from the "Sequence" menu.
2. Open "Sequence Table" from the "Sequence" menu.
3. Open "Insert/FillDown Wizard".
4. For "Starting location" enter "1" to indicate the location of your first vial.
5. For "Number of lines to insert" enter the total number of vials you are running (standards + blanks + samples)
6. For "Method name" enter "VFAISTD" for GC 1 or "3VFAISTD" for GC 3.
7. For "Inj./Location" enter "1" to indicate the number of times each vial will be sampled.
8. Scroll to the right, and enter "3" For "ISTD Amount" to indicate that each vial contains 3 mM of the internal standard.
9. For "Inj. volume" enter "1".
10. Leave other fields blank and press "OK".
11. "Cut" the first line if it was left blank.
12. Under "Sample Name" enter the individual sample name for each vial.
13. For every formic acid blank, change "Inj/Location" to "3" and change "ISTD Amount" to blank (Do not enter "0" or you will receive an error.)
14. Click "OK".
15. Open "Sequence Parameters" from the "Sequence" menu.
16. Enter your initials for "Operator Name".
17. Enter the folder name where you will find your data for "Subdirectory". We typically use initials followed by the date. (Ex: LH140428 for a run on April 28, 2014).
18. Click "OK".
19. A message will be displayed asking permission to create the subdirectory you have named. Click "OK".
20. If you will use the same or similar sequence in the future, save your sequence under "Save Sequence as" from the "Sequence" menu.

Starting Injections

1. Check that the method displayed on the main screen of the online program reads "VFAISTD.M" for GC 1 or "3VFAISTD.M" for GC 3.
2. Check that the box above "Start" and "Stop" buttons is green and reads "Ready".
3. Once it is ready, press "Start".
4. Wait for the GC to run through the syringe and needle cleaning process and inject the first blank before walking away. Most problems occur during this first injection.
5. If the computer displays a plunger error or injector error message, check that the plunger of the syringe moves freely and takes up and dispels water appropriately. Clean the needle and syringe again if necessary and restart the sequence.

6. If possible, create a standard curve and check for a good r^2 (usually > 0.995) as soon as the standards have finished running. (See **"Data Analysis"** for instructions.) This step allows potential errors to be identified before all samples have been run.

Shutting Down:

1. Close the "Instrument 1 (Online)" program on the computer, and turn off the communication module.
2. On the front of the GC, push the "Oven" button and turn the temperature off. Do the same for "Front Inlet" and "Front Det".
3. Once each component has cooled to at least 100° C, turn off the GC.
4. Turn off the helium, hydrogen, and compressed air cylinders ONLY if none of the other GCs are running.

Data Analysis

1. Open "Instrument 1 (offline)" for GC 1
2. Open "Load Signal" from the "File" menu.
3. Under the folders "HPCHEM", "1", and "DATA", find the folder with the subdirectory name that you created previously from "Sequence Parameters".
4. Select the first standard vial. Usually the file name will read "002F0201.D" because the first vial was a blank. The first number ("002") indicates the location number of the vial. Click "OK".
5. Check that the method displayed on the main screen is "VFAISTD.M"
6. Click the integration button. If an error or warning message is displayed, click "OK". Those errors will be fixed later.
7. Click the manual integration button.
8. Remove and unwanted peaks by dragging the mouse above the peak.
9. Select "New Calibration Table" from the "Calibration" menu.
10. For "Default Amount" enter the mM concentration of the first standard, usually "1". Click "OK". If asked to overwrite the existing calibration table, select "Yes".
11. On the table, name the compound for each peak. There should be 10 peaks that come out in the following order:
    1. Acetic acid (Ac)
    2. Propionic acid (Pr)
    3. iso-Butyric acid (iB)
    4. Butyric acid (Bu)
    5. iso-Valeric acid (iV)
    6. Valeric acid (Va)
    7. Internal Standard (ISTD)
    8. iso-Caproic acid (iC)
    9. Caproic acid (Ca)
    10. Heptanoic acid (Hep)
12. For peak #7 ("ISTD"), on the column named "ISTD" change the drop-down back from "No" to "Yes".

13. Click the blank box immediately to the right from the "#" column and enter "3" for "ISTD Amount". Click "OK". The remaining rows for the "#" column should fill in with "1" to indicate the ISTD that you have just named.
14. Load the next standard vial by selecting "Load Signal" from the "File" menu. The computer will automatically recognize the correct peaks identified in the calibration table, so steps 6 and 7 do not need to be repeated for the remaining vials.
15. Select "Add Level" from the "Calibration" menu.
16. Enter the concentration of the standard for "Default Amount" and click "OK".
17. Repeat steps 14 and 15 to load all standards.
18. At this point, the curves will look approximately like a straight vertical line because the ISTD concentrations are incorrect. On the calibration table, change the "Amt[mM]" values to "3" for all levels of the internal standard.
19. The computer will create a standard curve for each individual VFA. Check that all curves have a strong linear correlation (~ 0.995 or higher) by clicking on each compound name on the table.
20. Save the standard curve as a method. From the "File" menu, select "Save As" and then "Method". Change the method name to something you will recognize. We typically use the same name as the subdirectory (Ex: LH140428). Click "OK", leave "Comment for method history" blank, and click "OK" again.
21. From the "Batch" menu, select "Load Batch". In the right-hand box, select the subdirectory name you used for "Sequence Parameters". Open the folder "hpchem", "1" for GC 1 or "3" for GC 3, "data", and then double click the subdirectory name. In the box on the left click the batch name. There should only be one listed. It will either be called "DEF_GC.B" or another name that you gave it when creating the sequence. Click "OK".
22. Under "Method to Process Batch Data", click "Other Method", and select the method name that you just saved in step 20. Under "Select Runs for Batch Processing" click "Select All". Click "OK".
23. Wait for the batch to load. Click the "Start" button with a green arrow located towards the bottom of the screen.
24. Wait for the program to analyze each data file. Depending on the number of samples this may take 5-15 minutes. When the "Start" button is highlighted again, then the batch is finished.
25. From the "Batch" menu select "Output Batch Report" to export the data report to Excel.
26. The Excel report can be found under My Computer/Local Disk (C:)/ HPCHEM/ 1 or 3 (depending on GC used)/ DATA/ your subdirectory name (LH1404228). In that folder you will find a folder with data for each sample that was analyzed. At the end of those folders is an Excel file called "REPORT01.xls" that contains all of the analyzed results.
27. Open the Excel file. Select the "Labels" worksheet. Select cells E3:E25 and copy them. Select the "Data" worksheet. Select cell C1, "Paste Special", and "Transpose" to insert the correct column names. The first column for each VFA shows the retention time of the peak measured. The second column gives the concentration in mM calculated based on the created standard curves.

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